

**Loss of scribble causes cell competition in mammalian
epithelial cells**

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Declaration

I, Mark James Norman, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Cancer is a disease caused by transformation of cells by the activation or over-expression of oncogenes such as Ras and c-myc, and the loss of tumour suppressor genes such as E-cadherin and scribble. The initial stage of tumourigenesis is the transformation of a single cell in an otherwise normal epithelium. What occurs at this stage is largely unknown-do the transformed cells and normal cells co-exist or is there an antagonism between them? This thesis examines the fate of epithelial cells that lose the tumour suppressor scribble when in an otherwise normal epithelium.

The fate of scribble knockout clones has been studied in *Drosophila melanogaster* larval imaginal discs. It has been observed that scribble knockout clones are removed from the larval tissues by c-Jun N-terminal kinase (JNK) dependent apoptosis. It is thought that this is an innate tumour suppressive mechanism. It is therefore of great interest and importance to understand if a similar phenomenon can be seen in mammalian cells.

Scribble knockdown Madin-Darby canine kidney (MDCK) epithelial cells die only when surrounded by normal MDCK cells. Dead scribble short-hairpin RNA (shRNA) cells are apically extruded from the epithelium after cell death and exhibit classical apoptotic markers such as cytoplasmic condensation, caspase 3 activation and DNA fragmentation. Extrusion of dead scribble knockout cells occurs after initiation of apoptosis as blocking myosin activation results in many dead scribble knockout cells staying in the epithelial monolayer. Prior to cell death they maintain normal cell-cell adhesion with their normal MDCK neighbours and activate the stress induced protein kinase p38, but not c-Jun N-terminal kinase (JNK).

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All movies are on the attached CD.

Chapter 1. Introduction

Chapter 1 Introduction

1.1 Introduction and study concept

It is known that most cancers are caused by the accumulation of mutations in oncogenes, such as Ras or Myc, and tumour suppressors, such as p53 and retinoblastoma protein, in epithelial cells (Stratton et al., 2009, Hanahan and Weinberg, 2000). It is difficult to detect tumours until they are at quite an advanced stage, consisting of approximately 10^9 cells (Moreno, 2008). By this late stage of tumour development, tumour cells have very unstable genomes and can contain hundreds of mutated genes (Sjoblom et al., 2006, Greenman et al., 2007, Stratton et al., 2009). Early detection of cancer is something that clinicians and researchers have been targeting for many years, as it is associated with a much more optimistic prognosis.

In theory, the first step of tumourigenesis is the transformation of a single epithelial cell in an otherwise normal epithelium. This transformation could occur due to over-expression of an oncogene, or loss of a tumour suppressor. Previous work by the Fujita lab and others has shown that there are interesting, putatively tumour suppressive, phenotypes that occur when transformation occurs in a single epithelial cell in an epithelial monolayer (Kajita M et al., 2010, Hogan C et al., 2009, Igaki et al., 2009, Brumby and Richardson, 2003, Moreno, 2008). As well as deepening our understanding of tumourigenesis, it hoped that the study of the initial stages of tumourigenesis may lead to novel markers of transformation that can be used to detect tumours in their infancy. To fully understand these interactions between transformed and non-transformed epithelial cells, it is important to understand their conservation across species; many of these phenotypes have been solely characterised in *Drosophila melanogaster*. It is therefore the aim of this thesis to examine the fate of a small number of epithelial cells that have reduced expression of scribble, a tumour suppressor, in an epithelial monolayer.

1.2 What is scribble?

Scribble was first identified in 2000 as a mutant that disrupted epithelial morphogenesis in *D. melanogaster* embryos. The messy, disorganised nature of the embryonic cuticle in mutant embryos lead to the gene being named scribble (Bilder and Perrimon, 2000). The epidermis of *scribble*¹ mutant embryos was disorganised, showing multi-layering and poor cellular adhesion, suggesting a fundamental role for scribble in epithelial morphogenesis and maintenance (Bilder and Perrimon, 2000). *Scribble* mutant follicle cells (part of the follicular epithelium that surrounds developing *D. melanogaster* germline cells) and wing imaginal discs in particular, also showed severe morphological and polarity disruption, underlining the importance of scribble in epithelial homeostasis and its classification as a polarity protein (Zeitler et al., 2004, Bilder et al., 2000b).

Drosophila scribble is a large (1,756 amino acids in *D. melanogaster*) scaffold protein containing 16 amino terminal leucine rich repeats (LRR), two LAP specific domains and four PSD-95, Dlg, ZO-1 homology (PDZ) domains located towards the carboxyl terminus of the protein (Bilder and Perrimon, 2000, Santoni et al., 2002). In *D. melanogaster*, scribble protein localises to the septate junction, which is analogous to the mammalian tight junction and lies at the border of the apical and basolateral membranes (Bilder and Perrimon, 2000). Scribble localises to membranes, but is an intra-cellular protein and contains no trans-membrane domains (Bilder and Perrimon, 2000). Due to containing LRR and PDZ domains, scribble belongs to the LRR and PDZ (LAP) protein family, along with Densin 180, Erbin and Lano (Bilder et al., 2000a). The LRR repeats are able to localise to the plasma membrane alone and are essential for proper membrane localisation of scribble in mammalian cells and in *D. melanogaster* (Navarro et al., 2005, Zeitler et al., 2004). In fact, a single amino acid substitution (in mammalian cells, P305L, in *D. melanogaster* L223Q) in the LRR can render

¹ Several mutations of *scribble* have been created in *D. melanogaster*. The most commonly used allele is *scribble*¹. This contains a mis-sense point mutation (L223Q) and is a genetic null (Bilder and Perrimon 2000, Zeitler et al 2004). In this thesis, the phrase “*scribble* mutant” refers to complete loss of scribble function.

scribble unable to localise to the plasma membrane and functionally inactive (Zeitler et al., 2004, Navarro et al., 2005). The role of the PDZ domains is less clear; they are not required for rescue of polarity defects in scribble mutant tissues, but at least some of them (along with the LRR) are required to suppress the over-proliferation phenotype caused by loss of scribble (Zeitler et al., 2004). Many protein-protein interactions mediated by the PDZ domains (summarised in Table 1) are likely to be responsible for scribble's involvement in a wide-range of biological processes and signalling events (Nagasaka et al., 2010).

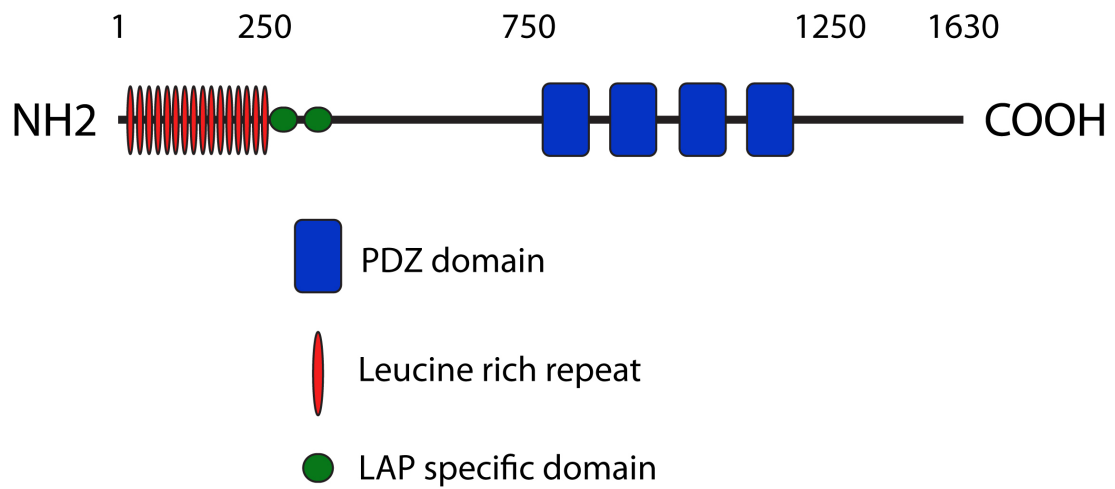


Figure 1 Protein map of human scribble. Sixteen leucine rich repeats (LRR) are located at the amino terminus, followed by two LAP specific domains of unknown function. Four PDZ domains are found between amino acids 750-1250. NH2 identifies the amino terminus of scribble protein and COOH the carboxy terminus. Numbers above the protein map show approximate amino acid positions.

A mammalian homologue of scribble (hScrib) was discovered by Dow et al in 2003 (Dow et al., 2003). Unlike other members of the Scribble/Lgl/Dlg complex (introduced in the next section), only one mammalian homologue of scribble has been identified in mammalian cells, making scribble a good protein to study the functions of this complex in mammalian systems. Human scribble is slightly shorter than its *D. melanogaster* homologue, consisting of 1630 amino acids (compared to 1756 amine acids in *D. melanogaster*) (Dow et al., 2003). It shares 37% total amino acid sequence with *D. melanogaster* scribble, with homology markedly higher in the highly conserved LRR and PDZ regions (Dow et al., 2003). Expression of GFP-hScrib in *D. melanogaster scribble* mutant clones rescues the

mutant phenotype, demonstrating the functional conservation of the proteins (Dow et al., 2003).

Scribble is a multi-function protein that has been shown to have roles in epithelial morphogenesis, planar cell polarity (Montcouquiol et al., 2003), cell-cell contact formation (Qin et al., 2005b), proliferation control (Zeitler et al., 2004, Brumby and Richardson, 2003, Brumby et al., 2004), mammalian embryogenesis (Murdoch et al., 2003), zebrafish neural tube extension (Wada et al., 2005) and gastrulation (Vervenne et al., 2008), polarised cell migration (Dow et al., 2007, Osmani et al., 2006), synaptic vesicle localisation (Sun et al., 2009), mammalian acini formation and tumourigenesis (Zhan et al., 2008) and cell competition (Brumby and Richardson, 2003, Igaki et al., 2009). I will now focus in detail on aspects of scribble cell biology that are relevant to work presented in this thesis.

Table 1 A summary of proteins that have been shown to directly or indirectly interact with scribble protein

Protein	Co-IP method	Reference
Dlg	Endogenous proteins	Mathew, 2002
Lgl	Over-expressed proteins	Kallay, 2006
Vangl2	Co-IP of endogenous scribble by over-expressed Vangl2	Kallay, 2006
Vimentin	Endogenous proteins	Phua, 2009 (Ivanov et al., 2010)
ZO-1	Endogenous proteins	Sun, 2009 (Arnaud et al., 2009)
β -catenin	Endogenous proteins (neurons)	
MCC	Co-IP of endogenous MCC with over-expressed scribble	Petit, 2005 p
Lipoma preferred Protein (LPP)	Recombinant protein pulldown	
β -PIX	Endogenous proteins	Audebert, 2004
GIT1	Endogenous proteins	Audebert, 2004

NOS1AP
ERK

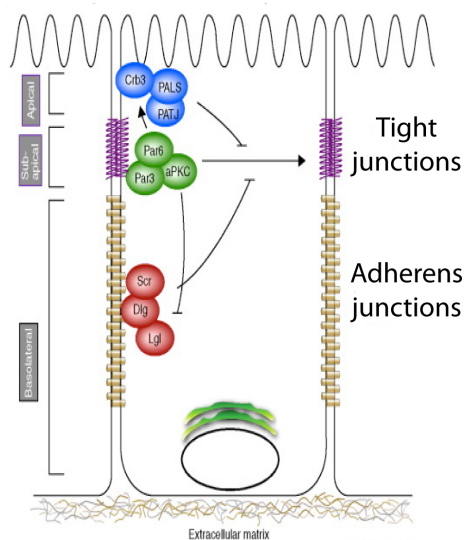
Endogenous proteins (neurons)
Over-expressed proteins

Richier, 2010
Nagasaka, 2010

1.3 Polarity proteins in epithelial cells

Epithelia cells form a barrier and are line the outside, and inside, of organs. They contain distinct apical and basal poles, and also have other domains such as the sub-apical and basolateral regions. The molecular mechanism of the establishment and maintenance of polarity in epithelial cells is very complex, but is centred around three complexes: the Crumbs complex which localises to the apical membrane, the Par complex which localises to the sub-apical region, and the Scribble complex which is found at the basolateral membrane in mammalian epithelial cells, and the septate junction (analogous to the mammalian tight junction) in *D. melanogaster* epithelial cells (the Scribble complex will be described in section 1.4) (Bulgakova and Knust, 2009, Humbert et al., 2008b, Humbert et al., 2006).

Mammalian epithelial cells



D. melanogaster epithelial cells

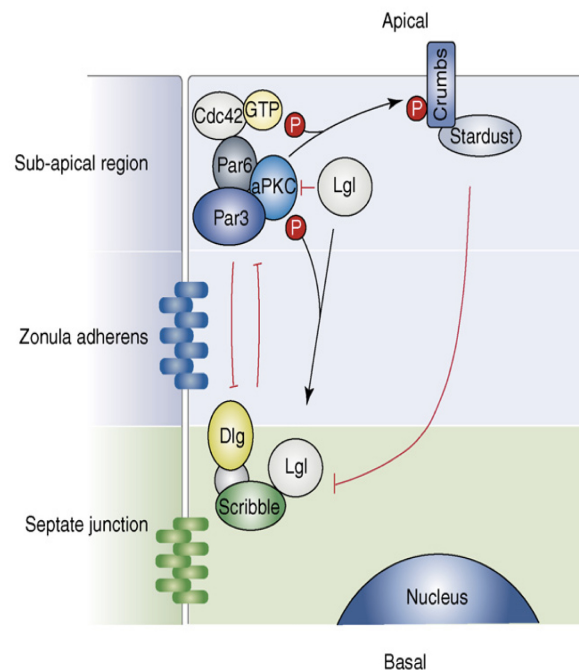


Figure 2 Key players in the polarity of mammalian and *D. melanogaster* epithelial cells. The distribution of key regulators of epithelial cell polarity in

mammalian (left) and D. melanogaster epithelial cells. Adapted from (Feigin and Muthuswamy, 2009, Humbert et al., 2006) (right image).

As well as the Scribble complex, the precise location of the Par is slightly different between mammalian and *D. melanogaster* epithelial cells. In mammalian cells it is localised to the tight junction, whereas in *D. melanogaster* it is localised apical to the fly version of the tight junction, the septate junction. E-cadherin based cell-cell contacts are also localised differently; in mammalian cells they are found on the whole length of the basolateral membrane, but in *D. melanogaster* epithelial cells they are specified to a point apical to the septate junction (Humbert et al., 2006, Feigin and Muthuswamy, 2009). These differences aside, the molecules and interactions between these complexes are highly conserved between species.

1.3 The Scribble complex

Scribble is grouped together into a complex with two other proteins, Lethal giant larvae (Lgl) and Discs Large (Dlg), that share many phenotypes and functions but little sequence homology. In *D. melanogaster* epithelial cells, scribble co-localises to the lateral membrane with Lgl and Dlg. Given this similarity in function, it is interesting to note that both Dlg and Lgl were discovered and characterised many years before scribble (Woods and Bryant, 1989, Mechler et al., 1985). A major function of this complex is to restrict the Par complex to its apical localisation (Humbert et al., 2006). Together, these two complexes significantly contribute to the control of apical-basal polarity.

Deletion in any one of the scribble, Lgl or Dlg genes causes massive overgrowth in *D. melanogaster* imaginal discs; consequently, they have all been termed *Drosophila* neoplastic tumour suppressor genes (nTSG). Deletion of one member of the complex causes the others to be mislocalised or lost (Bilder et al., 2000b). This genetic interaction and phenotypic correlation have led these three proteins to be grouped together into the Scribble/Lgl/Dlg complex, and it is presumed

that they are involved in common pathways (Humbert et al., 2008b). Despite the genetic evidence linking them together, biochemical evidence of the complex is less definitive. Co-immunoprecipitation (Co-IP) of endogenous scribble and endogenous Lgl has never been demonstrated; a small amount of scribble is able to co-IP with exogenous Lgl in MDCK cells (Kallay et al., 2006). The evidence for a physical interaction between Dlg and scribble is more concrete, with endogenous scribble able to co-IP with endogenous Dlg in *D. melanogaster* muscle wall extracts (Mathew et al., 2002). Such an interaction has, to my knowledge, not been demonstrated in mammalian cells.

1.4 Scribble in epithelial architecture

As introduced above, scribble has a vital role in maintenance of epithelial architecture in *D. melanogaster*. The molecular mechanism of apical-basal polarity control by scribble is not as well understood as for Lgl or Dlg (Humbert et al., 2006). Analysis of an array of *scribble* mutants in *D. melanogaster* has shown that the LRR are predominantly responsible for epithelia architecture; re-expression of only these domains is able to give a good, although not complete, rescue of the *scribble* mutant phenotype (Zeitler et al., 2004). In *scribble* mutant cells there is a mislocalisation of apical proteins armadillo (β -catenin) and crumbs, and the apico-lateral protein coracle, away from the apical membrane into the basolateral membrane (Bilder et al., 2003, Bilder and Perrimon, 2000, Zeitler et al., 2004). Despite this mislocalisation, the *scribble* mutant phenotype is not dependent on crumbs, as *scribble*/crumbs double mutants show similar phenotypes to *scribble* single mutants (Leong et al., 2009). Interestingly, the overgrowth and loss of epithelial architecture phenotype seen in clones of *scribble* mutant cells can be blocked by overexpression of a kinase dead, membrane tethered dominant negative of atypical protein kinase C (aPKC^{CAAXDN}) (Leong et al., 2009). This is consistent with data showing that loss of aPKC in *Lgl*^{-/-} mutant cells suppresses the *Lgl* mutant phenotype (Rolls et al., 2003). No defects in aPKC localisation can be seen in a *scribble* hypomorph (*scrib*⁴) in which over-proliferation and loss of epithelial structure phenotypes

were observed (Zeitler et al., 2004), suggesting that the relationship between scribble and aPKC is not straightforward.

The role of scribble in regulation of apical-basal polarity and epithelial morphogenesis in mammalian cells is less clear than in *D. melanogaster*. The localisation of scribble in mammalian cells is also slightly different to that in *D. melanogaster*. Mammalian scribble localises to the basolateral membrane and co-localises with Dlg, as in *D. melanogaster* (Dow et al., 2003, Bilder et al., 2000b). They differ in that mammalian scribble localises basal to the tight junction protein ZO-1 and overlaps with the adherens junction protein E-cadherin (Dow et al., 2003). When cultured on plastic substrates at low density, mammalian epithelial cells expressing an anti-scribble shRNA are flat, losing their epithelial morphology (Qin et al., 2005b). This phenotype, however, is not seen in all epithelial cell lines (Dow et al., 2007). In adhesion assays, MDCK cells with scribble knocked down by shRNA, have reduced cell-cell adhesion. This is likely to be due to improper localisation of E-cadherin to the cell-cell contacts, as levels of E-cadherin protein are not affected by knockdown of scribble (Qin et al., 2005b). Scribble is also required for efficient tight junction assembly; the time required for ZO-1 to re-localise to cell-cell contacts in a calcium switch assay is increased when scribble is knocked down (Qin et al., 2005b).

No disruption in apico-basal polarity in mammalian epithelial cells lacking scribble has been shown in conventional 2D culture. These studies are a poor reproduction of those in *D. melanogaster*, though, as culture in 2D is very different from physiological conditions. When cultured in Matrigel™ or type I collagen matrix, some mammalian epithelial cell lines (notably human MCF10A cells and canine MDCK cells, respectively) form 3 dimensional cysts or acini that are used as a more organotypic assay of epithelial cell morphogenesis and polarity (O'Brien et al., 2002). Even in this more physiological assay, knockdown of scribble has little effect on polarity. Scribble-knockdown MCF10A cells are still able to form 3D acini with correct apical-basal polarity, although there is a mild defect in orientation of the Golgi apparatus and a significant suppression of apoptosis that is thought to be required for proper acini formation (Dow et al.,

2007, Zhan et al., 2008). Scribble is, however, required for proper formation of acini structures in cleared mouse mammary fat pads, an *in vivo* assay for epithelial acini formation. In this assay, cells in which scribble is knocked down form poor acini with multilayered epithelia and poor lumen formation (Zhan et al., 2008). This assay reveals a requirement for scribble in mammalian epithelial morphogenesis similar to that in *D. melanogaster*, illustrating a role for scribble in this process conserved across species. The failure of more routine cell culture assays to demonstrate this role shows that there are limitations to these methods.

1.5 Scribble in cell migration

Outside of a role in polarity, scribble has an important role in cell migration. This feature of scribble biology has mostly been described in studies in mammalian cells, but was originally identified in *D. melanogaster* (Bilder et al., 2000b). In *D. melanogaster*, dorsal closure of embryos is commonly used as an assay of collective cell migration (Jacinto et al., 2002). In most cases, *scribble* zygotic mutants close properly and embryos survive until pupal stages. However, *scribble* mutants that lack a *Dlg* allele (*Dlg*^{+/-}) completely fail to close, showing that scribble and Dlg co-operate in collective cell migration in dorsal closure (Bilder et al., 2000b). This co-operation may be due to scribble localising Dlg to the leading edge, as this has been shown in mammalian astrocytes (Osmani et al., 2006). PAK (p21 activated kinase) is required for localisation of scribble to the leading edge in dorsal closure (Bahri et al., 2010). The relationship between scribble and PAK is bi-directional, as scribble binds PAK and is required for recruitment of PAK to the leading edge in mammalian cell culture assays of cell migration (Nola et al., 2008).

Analysis of scribble in mammalian cells led to the discovery that scribble binds β PIX, a guanine nucleotide exchange factor (GEF) for the Rho family GTPases Rac and cdc42. Scribble also binds GIT1, a GTPase activating protein (GAP) (Audebert et al., 2004). Scribble localises to the leading edge of migrating mammalian cells

and through β PIX, localises active Rac to the leading edge, propelling cells forward (Dow et al., 2007, Osmani et al., 2006). The affect of scribble knockdown on cell migration is variable depending on cell line. Knockdown of scribble in MDCK cells causes an increase in cell motility and invasion, and also a loss of persistence in wound closure assays; the sum of these phenotypes is that there is no defect in wound closure (Qin et al., 2005b). In contrast, similar experiments in MCF10A cells show scribble knockdown cells have no active Rac or cdc42 at the border of a wound, and consequently there is a marked reduction in wound closure (Dow et al., 2007). This study also showed that knockdown of scribble causes a defect in directed migration to a chemotactic cue. To further confirm this data, Dow et al used an *in vivo* wound healing assay in the rumpelschiltzchen mouse (*rumz*), which carries a mutation in the LRR of scribble. This assay also showed a failure of wound healing in *rumz*^{-/-} embryos, compared to *rumz* heterozygous or wild-type embryos.

There have been further studies of scribble mutants in mice (Murdoch et al., 2003) (the circletail strain) and zebrafish (Wada et al., 2005). The circletail mouse expresses a mutant form of scribble that does not have the two PDZ domains closest to the C-terminus (Murdoch et al., 2003). This strain has a defect in neural tube closure, which requires convergent extension of a cell sheet (Murdoch et al., 2003). The scribble mutant zebrafish also shows defects in convergent extension, as well as neuronal migration (Wada et al., 2005). Taken together with data from *D. melanogaster* and cultured mammalian cells, these data show a requirement for scribble in migration of epithelial cell sheets across species.

1.6 Scribble and proliferation

One of the initial phenotypes of scribble detailed the overgrowth of *scribble* whole mutant wing discs (Bilder et al., 2000b); further work in *D. melanogaster* has also shown that *scribble* mutant cells proliferate aberrantly (Brumby and Richardson, 2003). A failure to differentiate and a failure in tissue size control

may contribute to this phenotype (Johnston and Gallant, 2002). More specifically, scribble has also been identified in a screen for suppressors of a cyclin E hypomorph mutation in *D. melanogaster* eye imaginal discs, suggesting that scribble regulates entry into S phase (Brumby et al., 2004). Loss of scribble in *D. melanogaster* follicle cells also increases proliferation, as measured by BrdU uptake and the presence of phosphorylated histone H3 (Zhao et al., 2008). These data confirm that scribble negatively regulates cell proliferation in *D. melanogaster*.

There is evidence that scribble also regulates cell proliferation in mammalian cell lines. Over-expression of scribble in epithelial (MDCK, HeLa) or fibroblast (NIH3T3) cells suppresses proliferation, with the cell cycle arresting in G1. Knockdown of scribble in Caco-2 epithelial cells decreases the percentage of cells in G1 and increases the percentage in S phase. These data confirm a role for scribble in regulating entry into S phase (Nagasaka et al., 2006). Scribble regulates ERK activation in mammalian cells; knockdown of scribble increases phosphorylation of ERK and translocation to the nucleus. Scribble is also phosphorylated by ERK, showing that these proteins have a dual-regulatory relationship; it is not currently known how phosphorylation may affect the function of scribble protein (Nagasaka et al., 2010). An independent study has also shown that loss of scribble in MCF10A cells causes an enhanced increase in phospho-ERK levels upon EGF stimulation (Dow et al., 2008). Despite these reports in *D. melanogaster* and the regulation of ERK by scribble, there are no reports that scribble knockdown causes over-proliferation in mammalian cells. Scribble knockdown in MCF10A acini does not inhibit proliferation arrest required for correct acini formation (Zhan et al., 2008). Studies of scribble mutant animals do not mention any overgrowth phenotypes similar to those seen in *D. melanogaster* (Murdoch et al., 2003, Wada et al., 2005). Further work is required, then, to clarify the role of scribble in proliferation control in mammalian cells.

1.7 Scribble in human cancer and cancer models

From its first discovery, the similarity of *scribble* mutant phenotypes to those of the other *Drosophila* neoplastic tumour suppressor genes led to a presumption that scribble was a tumour suppressor (Bilder et al., 2000b). In the intervening ten years there have been many studies showing a role for scribble in many cellular processes that are important in cancer, such as cell migration, adhesion, differentiation and proliferation. The genetic tools to study scribble in mouse models of tumourigenesis do not yet exist. The development of these will be crucial for advancing understanding of the role of scribble as a tumour suppressor in cancer. Despite these technical limitations, clinical studies and one mouse study have given insight into the importance for scribble as a mammalian tumour suppressor.

Interestingly, scribble, like many other proteins that contain a PDZ domain, is targeted for degradation by the human papilloma virus (HPV) oncoproteins E6 and E7 (Nakagawa and Huibregtse, 2000). HPV is a driving force behind several cancers, in particular cervical cancer where it is found in 80% of cases (Thomas et al., 2008). HPV containing the E6 oncoprotein causes more aggressive tumours than those caused by E7 containing HPV (Thomas et al., 2008). E6 oncoprotein binds scribble, resulting in ubiquitination of scribble and its targeting for proteasomal degradation (Nakagawa and Huibregtse, 2000). Consequently, in cervical cancer the level of scribble protein decreases with increased malignancy (Nakagawa et al., 2004). HPV E6 affects a large number of proteins, and in particular a large number of polarity proteins, so it is difficult to determine the specific requirement for the loss of scribble in HPV infected tumours (Thomas et al., 2008). One study has shown that over-expression of scribble in cell culture suppresses colony formation in cells that contain the E7 oncoprotein and oncogenic RasV12 (Nagasaka et al., 2010). These data suggest that loss of scribble is a vital requirement for the oncogenic role of HPV and that loss of scribble may be one of the key mediators of tumourigenesis in HPV infected cells.

Immunohistochemical analysis of human breast (Navarro et al., 2005) and colon cancer (Gardiol et al., 2006) tissue samples have shown that scribble is lost

during malignant progression. In these cases there were other polarity proteins also lost, so it cannot be said whether there it is the general loss of polarity or the specific loss of scribble that is required for malignant progression. Confusingly, the chromosomal region that harbours the scribble gene (8q24.3) is amplified in a wide range of cancer cell lines, breast tumours and ovarian tumours (Naylor et al., 2005, Kim et al., 2007). Deletion of this region was also reported, but the amplification was seen a lot more frequently (Naylor et al., 2005). A mutation in the LRR of scribble can cause the same defects as deletion of the protein (Zeitler et al., 2004), so it is important to analyse the sequence of the scribble gene in these tumours where 8q24.3 is amplified to identify any mutations in the scribble gene. Scribble has been shown to accumulate in colorectal tumours, and correctly localises to the cell membrane suggesting that the protein is wild-type, although correct localisation is not conclusive evidence that it is of wild-type function (Kamei et al., 2007). Another study of scribble in human cancer shows that scribble expression in tumours can be very heterogeneous, with loss of the protein in some regions but expression of the protein in another; this may explain why scribble can be found at high levels in some tumours (Zhan et al., 2008). In regions that scribble expression is maintained, it is often mislocalised (Zhan et al., 2008). This suggests that scribble may be mutated in these regions where expression is maintained. Taken together these data show that scribble is lost or mutated in some human tumours. Like many proteins involved in cancer, the role of scribble in tumours is likely to be dependent on cell type and other mutations in the tumour.

To date there has only been one examination of the role of scribble in a murine cancer model. In this study, knockdown of scribble was used to analyse the effect of loss of polarity on tumourigenesis. Scribble was knocked down in cells that over-express c-Myc (MycOE:Scrib KD cells), before transplantation of cells into cleared mammary fat pads of mice. The oncogene c-Myc was chosen in this study as it does not induce polarity defects. MycOE:ScribKD cells produced detectable tumours quicker than MycOE alone, and the resultant tumours were larger, with less apoptosis and less differentiation than tumours formed by cells over-expressing Myc only (Zhan et al., 2008). Consequently, a reduction in survival

time was seen in mice with MycOE:Scrib KD driven tumours compared to those with MycOE driven tumours (Zhan et al., 2008). In addition, scribble was frequently lost or mislocalised in Myc driven mammary tumours in transgenic mice and in human breast tumour samples (Zhan et al., 2008). These data suggest that loss of scribble aids tumour progression and support the role for scribble as a mammalian tumour suppressor.

1.8 Cell competition

Another significant focus of scribble research is on the role of scribble in cell competition. Cell competition is a term used to describe a phenomenon where cells that are expressing different levels of a particular protein are somehow able to recognise this difference in protein level, resulting in death of one population of cells (said to be the loser population) and survival of the other cell population (Moreno, 2008). Essential to the concept is that both populations are viable when they are surrounded by cells genetically identical to themselves. It is thought to be a homeostatic mechanism to ensure proper tissue growth (Johnston, 2009).

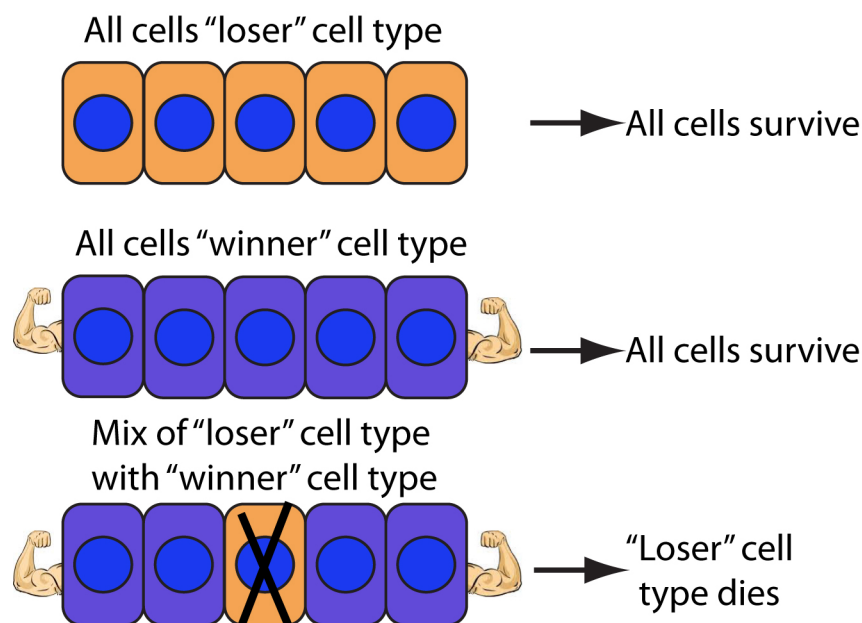


Figure 3 Central concept of cell competition. Two cell types, for simplicity called "winner" and "loser", are both viable when with cells genetically identical to

themselves. When loser cells are confronted with winner cells, the loser cells are eliminated by apoptosis. The loser cells or winner cells may be wild-type or mutant, depending on the particular example of cell competition (see text for details).

Cell competition was originally discovered in *D. melanogaster* in the 1970s by Morata and Ripoll (Morata and Ripoll, 1975). This original study focused on cells mutant for *Minute*, a mutation that results in lower ribosomal activity. *Minute* cells proliferate slower than wild-type but are viable, and *Minute* heterozygous animals develop to adulthood. When clones of *Minute* heterozygous (*Minute/+*) cells are made in a wild-type epithelium, *Minute/+* cells are removed from the epithelium (Morata and Ripoll, 1975). It was discovered many years later that the removal of *Minute/+* cells was due to apoptosis (Moreno et al., 2002).

Mutants of the decapentaplegic (Dpp) receptor Thickvein (Tkv) were the second class of mutations that were described to induce a cell competition phenotype in *D. melanogaster*. Tkv is the receptor for Dpp, a member of the TGF- β superfamily (Raftery and Sutherland, 1999). Together, Dpp and Tkv produce a controlled gradient of signalling activity that is required for correct patterning of many tissues in *D. melanogaster*, including the wing imaginal disc (Lecuit et al., 1996, Nellen et al., 1996). Despite the importance of Dpp signalling, wing discs where every cell expresses a *tkv* hypomorph develop with relatively minor abnormalities; however, when clones of *tkv* hypomorph cells are induced in wild-type wing discs, they are removed by apoptosis (Adachi-Yamada et al., 1999). The elimination of *tkv* hypomorphs was suppressed by reducing the level of JNK activity in the wing disc; JNK activity was to become a central theme in future cell competition research (Adachi-Yamada et al., 1999). Interestingly, over-expression of a constitutively active version of the Tkv receptor also resulted in death of these cells, and some neighbouring wild-type cells (Adachi-Yamada et al., 1999, Adachi-Yamada and O'Connor, 2002). This led to the appealing hypothesis that these mutants disrupted the usual smooth gradient of Dpp signalling in the wing disc and that cells that express inappropriate levels of Dpp signalling, be it high or low, died in order to restore the usual gradient of Dpp signalling; thus this phenotype was called “morphogenetic apoptosis” (Adachi-

Yamada and O'Connor, 2002). Work by others has shown that the induction of cell death due to sharp boundaries of Dpp signalling is required for proper development of leg tissue in *D. melanogaster*, showing that this phenomenon occurs in a natural as well as experimental setting (Manjón et al., 2007). It is important to note that in morphogenetic apoptosis, cells on either side of the sharp boundary of Dpp signalling are eliminated, unlike in cell competition where only one cell type dies (Adachi-Yamada and O'Connor, 2002, Manjón et al., 2007, Moreno et al., 2002). Therefore, despite being mechanistically similar to cell competition caused by *Minute* mutation, this lack of a clear winner and loser cell population suggests that the upstream signalling pathways in these two processes may be distinct.

Shortly afterwards, it was discovered that the proto-oncogene Myc is able to induce cell competition. This caused a great deal of interest in cell competition and led to the proposal that cell competition could be related to cancer. Clones of cells that express a *Myc* hypomorph (*dmyc*^{P0}) die when surrounded by cells expressing a wild-type level of dMyc (*Drosophila Myc*) (Moreno and Basler, 2004, de la Cova et al., 2004). Furthermore, when clones expressing wild-type dMyc are induced in a background of dMyc over-expression, the cells with lower dMyc expression die (Moreno and Basler, 2004). These experiments show that there is a mechanism in developing tissues to eliminate cells that have lower Myc expression. When small clones of cells over-expressing dMyc are induced in a wild-type background they over-proliferate, as would be expected. Amazingly, dMyc over-expressing cells are also able to induce cell death in wild-type neighbours (de la Cova et al., 2004, Moreno and Basler, 2004). This induction of cell death is not solely due to the increased proliferation of the dMyc over-expressing cells as clones that over-express cyclin E over-proliferate but are not able to induce apoptosis in neighbouring wild-type clones (de la Cova et al., 2004). This induction of cell death by dMyc over-expressing cells means that the tissue retains its wild-type size, but becomes dominated by dMyc over-expressing cells (de la Cova et al., 2004, Moreno and Basler, 2004). Interestingly, this is reminiscent of field cancerisation, a theory of cancer development where a mutation in a single cell allows the mutant cells to colonise a tissue. It is thought

that a further mutation in these cells allows tumourigenic progression (Braakhuis et al., 2003).

Since these initial findings, several genes have been proposed to cause cell competition. Principal among these are genes of the scribble complex. Several studies in *D. melanogaster* have identified that scribble and Lgl mutants cause cell competition (Brumby and Richardson, 2003, Igaki et al., 2009, Tamori et al., 2010, Menendez et al., 2010, Froidi et al., 2010, Leong et al., 2009). In addition, loss of Mahjong/VprBP (Mahjong), an Lgl binding protein, also causes cell competition; it appears that Mahjong lies downstream of Lgl in cell competition, as over-expression of *Mahjong* in *Lgl* mutant cells rescues viability in these cells (Tamori et al., 2010). There are reports of *Dlg* mutant clones dying when surrounded by wild-type cells, but there is not yet convincing evidence that this is due to the interaction with neighbouring wild-type cells (Cordero et al., 2010). Other genes have been suggested to cause cell competition but have not been shown to fulfil the criteria specific for cell competition; specifically, the observed death in these cells has not been proven to rely on interaction with wild-type cells. It seems likely that in the future more genes that can cause cell competition will be identified.

Table 2 Summary of genes known to induce cell competition. Note that *Myc* and *Thickvein* have been shown to induce cell competition when their protein level/activity is reduced or increased relative to neighbours.

Mutation	Function of protein	Reference
<i>Scribble</i>	Cell polarity, tumour suppressor	Brumby, 2003
<i>Lgl</i>	Cell polarity, tumour suppressor	Tamori, 2010
<i>Mahjong</i>	Lgl binding protein	Menendez, 2010
<i>Minute</i>	Ribosomal proteins	Tamori, 2010
<i>Myc</i>	Transcription factor, oncogene	Morata, 1975
<i>Thickvein</i>	Dpp receptor	de la Cova, 2004
		Moreno, 2004
		Adachi-Yamada,

1.9 Scribble and cell competition

Along with *Minute* and *Myc*, scribble mutation is one of the best described examples of cell competition. The contrast between the two phenotypes, i.e. neoplastic overgrowth when all cells are *scribble* mutant and apoptosis when only a small number of cells are mutant, makes it a fascinating subject. Brumby and colleagues first described that clones of *scribble* mutant cells die and are eliminated from the *D. melanogaster* eye imaginal disc epithelium (Brumby and Richardson, 2003). The eye disc is a very good system to study putative tumour suppressors and oncogenes because it undergoes a progressive differentiation (Wolff and Ready, 1991). *Scribble* mutant clones induced in the eye disc have increased proliferation due to up-regulation of cyclin E, and also fail to differentiate (Brumby and Richardson, 2003). These are phenotypes that are consistent with the described function of scribble as a tumour suppressor. However, further examination of *scribble* mutant clones showed that they were significantly smaller than control GFP clones, and mosaic WT/*scribble* eye discs contained many apoptotic cells. Expression of the caspase inhibitor p35 in *scribble* clones increased the size of the clones, but the most dramatic rescue was by over-expression of a dominant negative version of the *D. melanogaster* homologue of JNK (Basket DN) (Brumby and Richardson, 2003). This led to the conclusion that *scribble* mutant clones were removed from the epithelium by JNK induced apoptosis. Simultaneous induction of *scribble* clones and removal of the wild-type cells by over-expression of the caspase inducer head involution defective (*hid*) gave very large clones, confirming that the induction of cell death in *scribble* mutant clones required the surrounding wild-type tissue (Brumby and Richardson, 2003). These studies demonstrate superbly that *scribble* mutant clones die when surrounded by normal epithelial cells, but are viable when surrounded by other *scribble* mutant cells.

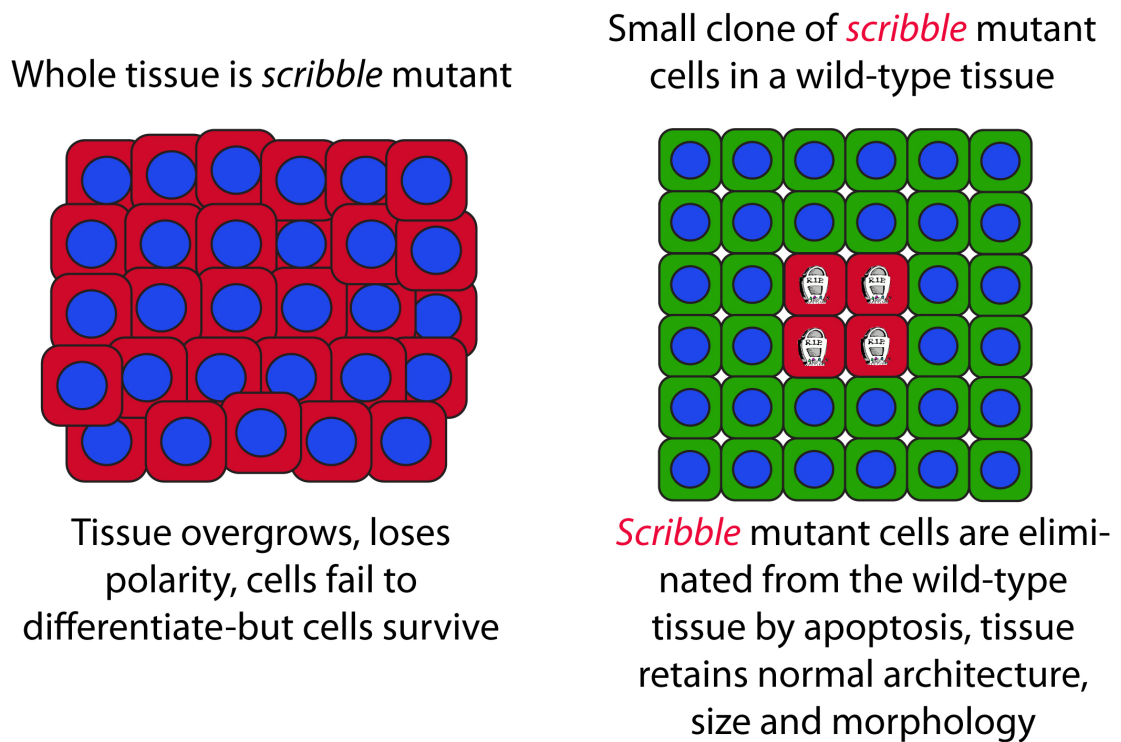


Figure 4 Summary of contrasting *scribble* mutant phenotypes. When *scribble* is lost in all cells of a tissue, cells over-proliferate and lose polarity (left image). When a small clone of *scribble* cells are induced in a wild-type epithelium, they die (right image).

Further studies by Igaki et al led to greater understanding of how *scribble* mutant clones *D. melanogaster* are removed from the epithelium. Igaki et al showed that the removal of *scribble* mutant clones from the eye imaginal disc epithelium is dependent on Eiger, the *D. melanogaster* homologue of tumour necrosis factor alpha (TNF α) (Igaki et al., 2009). Clones of *scribble* mutant cells survive when induced in flies that do not express *Eiger*, resulting in abnormal pupal development. Very low levels of Eiger protein are present in *scribble* mutant clones, but *scribble/Eiger*^{-/-} double mutant clones may have increased survival, suggesting that *scribble* mutant clones may be the source of Eiger (Igaki et al., 2009). These data are consistent with other reports, as Eiger is known to activate JNK signalling which is required for elimination of *scribble* clones (Brumby and Richardson, 2003, Igaki et al., 2002). Interestingly, *scribble* mutant clones also have increased endocytosis, as shown by increased uptake of fluorescent

Dextran and increased expression of GFP-tagged Rab5. This increase in endocytosis in *scribble* mutant cells requires the interaction with wild-type cells, as no increase in Dextran uptake is seen when wild-type tissue is removed from *scribble* mosaic tissue (Igaki et al., 2009). Furthermore, *scribble* mutant clones expressing Rab5 dominant negative, which reduces endocytosis, have increased survival (Igaki et al., 2009). These data are an interesting contrast to data showing that over-expression of Rab5 rescues cell competition caused by lowered dMyc expression (Moreno and Basler, 2004).

A study by Cordero et al has further clarified the role of TNF in removal of *scribble* mutant clones, and other mutants of the scribble complex. Adding to research by Igaki et al, they show that haemocytes are recruited to *lgl*^{-/-} and *Dlg*^{-/-} mutant clones, and that these haemocytes contain high levels of Eiger (Cordero et al., 2010). Previous work has shown that haemocytes are recruited to whole *scribble* mutant eye discs (Pastor-Pareja et al., 2008). Crucially, knockdown of Eiger by a haemocyte specific RNAi allows *lgl*^{-/-} clones to persist in the wing disc epithelium (Cordero et al., 2010). It is not shown explicitly in this study that knockdown of Eiger in haemocytes also allows *scribble* clones to survive, but it is implied by the authors that mutations in the scribble complex are interchangeable in this study. This is the first suggestion that the immune system is involved in cell competition. As haemocytes are recruited to *scribble* whole mutant tissue, it would seem that the increased endocytosis seen in *scribble* surrounded by wild-type cells is required for the induction of cell death in these clones by Eiger secreted from haemocytes (Pastor-Pareja et al., 2008, Igaki et al., 2009). These data show that elimination of *scribble* cells is due to co-operation between wild-type cells neighbouring cells and circulating haemocytes that are recruited to the *scribble* cells due to an autonomous event. Therefore, in *D. melanogaster* the elimination of *scribble* cells in a wild type tissue is due to a combination of autonomous and context specific events.

1.10 Co-operation of loss of scribble with oncogenes

How can scribble be a tumour suppressor if cells that are mutant for scribble are eliminated by wild-type tissue? Tumourigenesis is a multistep process and no oncogene or tumour suppressor is able to induce a full tumour alone (Hanahan and Weinberg, 2000). Several studies, mostly in *D. melanogaster* but also in mammalian cells, have shown that loss of scribble in a small number of cells is able to co-operate with oncogenes to give a dramatically transformed phenotype.

In a screen for metastatic behaviour in *D. melanogaster*, Pagliarini and Xu identified loss of scribble as a key step for metastasis of RasV12 expressing cells (Pagliarini, 2003). In this screen, clones of RasV12 cells over-proliferate and form tumours that remain in the tissue of origin (in this case the eye imaginal disc). These were termed *in situ* tumours. Deletion of scribble in RasV12 expressing clones massively increased the overgrowth phenotype of RasV12 expression and allowed mutant cells to leave the eye-antennal disc and invade nearby tissues such as the ventral nerve cord and those further away, such as leg imaginal discs (Pagliarini, 2003). Loss of scribble complex members Lgl or Dlg in conjunction with RasV12 expression also gave metastatic behaviour (Pagliarini, 2003). The metastatic phenotype, but not over-proliferation, of RasV12/*scribble* clones was suppressed by over-expression of E-cadherin (Pagliarini, 2003). These data places scribble mutation as a critical step in the progression of tumours to metastasis, a key step in the progression to malignancy.

A subsequent paper from the same lab interestingly proposed that RasV12 expression and *scribble* mutation does not have to occur in the same cell in order to give metastatic overgrowth. Induction of a RasV12 clone adjacent to a *scribble* mutant clone gave massive overgrowth in the eye-antennal disc and invasion to the ventral nerve cord-the same phenotype as double RasV12/*scribble* mutation in the same cell (Wu et al., 2010). Close inspection of these tumours shows that they mostly consist of GFP marked RasV12 cells; most *scribble* mutant cells are lost during the process of tumourigenesis (Wu et al., 2010). The authors theorise that JNK activation in *scribble* mutant clones is spread to the neighbouring

RasV12 expressing clone, and this increase in JNK signalling induces signalling through the JAK/STAT pathway which is required for tumourigenesis (Wu et al., 2010). To support this, inhibition of JNK signalling in the RasV12 expressing clones neighbouring *scribble* mutant clones suppresses invasion. Wounding is known to induce JNK activation that can spread away from the wound to induce compensatory proliferation necessary to close the wound (Bosch et al., 2005); it seems that in this scenario, the normal signalling induced to close the wound caused by the death of *scribble* mutant cells is required for invasion (Wu et al., 2010). It is known that expression of dominant negative JNK (*BasketDN*) in *scribble* mutant clones suppresses cell competition mediated death of *scribble* mutant cells (Brumby and Richardson, 2003, Igaki et al., 2009). It is disappointing, therefore, that the role of JNK in *scribble* mutant clones is not addressed in this study. It would be interesting to understand if the death of *scribble* mutant clones induced by cell competition is required for the invasion of RasV12 cells into other tissues, as this would propose a pro-tumourigenic function of scribble induced cell competition, which had been previously assumed to be an inhibitor of tumourigenesis (Igaki et al., 2009). Indeed, it has been shown that mechanisms used to remove *scribble* mutant cells can be subverted into a transformative mechanism (Cordero et al., 2010).

As described above, removal of deletion mutants of the scribble complex in *D. melanogaster* requires Eiger/TNF (Igaki et al., 2009, Cordero et al., 2010). Therefore, it was assumed that TNF activity in *scribble* clones was tumour suppressive. However, Cordero et al show that TNF is required for the invasive phenotype of RasV12/*scribble* mutant clones. As already described, RasV12/*scribble* double mutant clones are invasive (Pagliarini, 2003). To enable invasion, RasV12/*scribble* double mutant cells up-regulate matrix degrading enzymes (Cordero et al., 2010). When RasV12/*scribble* clones are induced in an *Eiger*^{-/-} mutant background, these clones overgrow but remain *in situ*, do not invade and do not up-regulate expression of matrix degrading proteins (Cordero et al., 2010). This suggests the tumour suppressive response induced to eliminate *scribble* clones is in fact required for invasion of double mutant

RasV12/*scribble* cells. It is very interesting that Eiger can have such different roles.

Significantly, co-operation between loss of *scribble* and oncogenic Ras has also been described in the mammalian epithelial cell line MCF10A. Dow et al present data that is very similar to that seen in *D. melanogaster*. Using acini formation of MCF10A as their model, they show that RasV12 over-expressing MCF10A cells form acini with no lumen, disrupted apical-basal polarity and with cells that do not exit the cell cycle compared to control acini (Dow et al., 2008). It has previously been described that knockdown of *scribble* in MCF10A cells gives no alteration in acini formation (Dow et al., 2007). Similar to studies in *D. melanogaster*, knockdown of *scribble* in RasV12 over-expressing MCF10A cells results in severely deformed acini that frequently produced long invasive spikes into the surrounding matrix (Dow et al., 2008). Invasive spike formation was mildly inhibited by inhibition of JNK or p38MAPK, but was almost completely blocked by inhibition of MEK-ERK signalling (mitogen activated protein kinase/extracellular-regulated kinase) (Dow et al., 2008). Raf is the key downstream effector of Ras in this process, as over-expression of active Raf in *scribble* knockdown MCF10A cells also gives a significant increase in invasion. This study identifies *scribble* as an important regulator of MEK-ERK signalling in mammalian cells and wonderfully reproduces RasV12/*scribble* oncogenic co-operation seen in *D. melanogaster*.

Finally, clones of *scribble* mutant cells in *D. melanogaster* can be rescued from death by over-expression of active Notch. This results in massive overgrowth of the mutant clone, but it has not been studied intensively so it is unknown whether this drives invasion (Brumby and Richardson, 2003). This study was also the first to identify that Ras activation in *scribble* mutant clones causes neoplastic overgrowth. Several other genes have been studied in conjunction with *scribble* mutation, including oncogenic β -catenin and phosphatidylinositol-3 kinase (PI3K), which do not co-operate with loss of *scribble*, showing that there is specificity in the synergistic action of these oncogenes with *scribble* mutant cells (Brumby and Richardson, 2003).

Taken together with data from experiments with scribble knockdown and over-expression of Myc already described, these reports demonstrate a conserved role for scribble in suppressing tumourigenic potential of different oncogenes. These interactions underline the role of scribble as tumour suppressor.

1.11 Molecular mechanism of cell competition

Recent studies of cell competition have begun to clarify the molecular mechanism of cell competition in *D. melanogaster*. There are two principle hypotheses proposed to explain cell competition.

Experiments in *D. melanogaster* have led to a theory called the ligand capture hypothesis (Moreno and Basler, 2004). It is known that in developing imaginal wing discs, cells are constantly competing for a limited supply of extra-cellular morphogens and growth factors and that disruption of this signalling results in cell death or apical extrusion (Shen and Dahmann, 2005, Moreno et al., 2002, Adachi-Yamada et al., 1999, Adachi-Yamada and O'Connor, 2002, Gibson and Perrimon, 2005). It has been proposed that cells that die in cell competition are less able to capture, internalise and transduce extra-cellular ligands into intra-cellular signalling events than their neighbouring cells (Johnston, 2009). In this hypothesis, cell death is a result of a passive process that does not necessarily involve direct communication between the two cell populations. In support of this theory, clones of *Minute* mutant cells in the wing imaginal disc have lower pMad signalling, indicating poor reception of Dpp ligand (Moreno et al., 2002). Over-expression of a dominant active form of the Dpp receptor, Thickvein^{QD} (Tkv^{QD}), in clones of wild-type cells in a Myc over-expression background increases survival of the clones, as does increasing the endocytic capability of the clone by over-expression of Rab5 (Moreno and Basler, 2004). These data support the hypothesis that outcompeted cells are unable to capture sufficient extra-cellular ligands. Myc controls expression of several genes required for ribosomal biogenesis, so Myc and Minute mediated cell competition may be mechanistically

similar (Grewal et al., 2005). Further evidence for a role of Dpp in cell competition comes from a screen for suppressors of death of *Minute* mutant cells (Tyler et al., 2007). Many of the suppressors identified in this screen restored Dpp signalling to wild-type levels, suggesting this is critical in order to allow *Minute* mutant cells to survive. A study in a *D. melanogaster* cell line has shown that cell-cell contact between Myc over-expressing cells and wild-type cells may not be required to induce cell death in the wild-type cells (Senoo-Matsuda and Johnston, 2007).

Contradicting this theory of competition for extra-cellular ligands, *scribble*/Rab5 dominant negative double clones have increased survival, showing that endocytosis can have a negative effect on clone survival. In addition, over-expression of Tfv^{QD} has no effect on *scribble* mutant clone survival (Brumby and Richardson, 2003, Igaki et al., 2009). These data suggest that Dpp signalling is not critical in the elimination of *scribble* mutant clones. The opposite requirement for endocytosis in *scribble* mutant cells compared to cells with lower dMyc expression means that it is unlikely that the ligand capture hypothesis can explain all types of cell competition.

Alternatively, local interactions between neighbouring competing cells may be responsible for cell competition. In many cases of cell competition, it has been shown that cells at the borders of the mutant clones, i.e. those that are directly contacting cells of the other population, undergo cell death (de la Cova et al., 2004, Tamori et al., 2010, Li and Baker, 2007). Excellent work on the role of engulfment in cell competition also supports a role for direct interaction between the two cell types in cell competition. Experiments in the nematode work *Caenorhabditis elegans* have shown that engulfment of cells can be required to push unhealthy cells into apoptosis (Hoeppner et al., 2001). Li and Baker show that proteins associated with engulfment of dead cells by glia or cultured *D. melanogaster* cells are required for the induction of cell death in outcompeted *Minute*/+ cells (Li and Baker, 2007). Cells mutant for *draper*, a trans-membrane receptor, or *wasp*, a regulator of actin dynamics, are unable to outcompete *Minute*/+ cells (Li and Baker, 2007). This is not simply a failure of

apoptotic cell clearance, as there is a reduction in caspase-3 positive *Minute/+* cells when they border *wasp* or *draper* minute cells rather than wild-type cells (Li and Baker, 2007). This shows that lack of survival signalling alone is insufficient to induce cell death in outcompeted clones. It now seems apparent that no single mechanism, be it active engulfment by neighbouring cells or lack of survival signalling, is entirely responsible for cell competition and that these processes are complementary, not contradictory.

Moving from external cues of apoptosis to inter-cellular signalling pathways, it is thought that JNK plays a central role in the induction of apoptosis in out-competed cells. Many cases of cell competition have shown that cell death is JNK dependent. This is true whether the initial cue is mutation of *scribble*, *Lgl* or *Minute* (Brumby and Richardson, 2003, Moreno and Basler, 2004, Tamori et al., 2010, Moreno et al., 2002, Igaki et al., 2009). The role of JNK in cell competition induced by different levels of dMyc is more disputed, with one group showing that cell death is dependent on JNK activity and another claiming inhibition of JNK only saves a fraction of outcompeted cells from cell death (Moreno and Basler, 2004, de la Cova et al., 2004). Also, the screen carried out by Tyler et al mentioned previously did not pull out any known members of the JNK pathway; this is surprising given the reported importance of JNK in death of *Minute* mutant cells that they use in their screen (Tyler et al., 2007, Moreno et al., 2002). Tyler et al propose that the heat shock method commonly used to induce clones in *D. melanogaster* may be responsible for activation of JNK, but de la Cova et al also use heat-shock induced methods and showed that JNK is not completely required for death of outcompeted cells (de la Cova et al., 2004). The requirement of JNK in cell competition, therefore, is not completely clear, but the number of experiments showing that inhibition of JNK increases survival of outcompeted clones does suggest an important role. The suppression of cell competition in mammalian cells by inhibition of JNK further supports an important role for JNK in cell competition (Tamori et al., 2010).

The most recent signalling pathway to be implicated in cell competition in *D. melanogaster* is the Salvador-Hippo-Warts pathway (abbreviated to the Hippo pathway). The Hippo pathway is a recently discovered pathway that regulates the cell cycle, apoptosis and organ size in *D. melanogaster* and mammals, and has an emerging role in cancer (Tapon et al., 2002, Pantalacci et al., 2003, Hariharan, 2006, Zeng and Hong, 2008, Dong et al., 2007). Yorkie is a transcriptional co-activator that is negatively regulated by the Hippo pathway; when the pathway is activated, Yorkie is inactive and localised to the cytoplasm (Huang et al., 2005). Menendez et al show that *Lgl* mutant clones in *D. melanogaster* wing discs are rescued by RasV12 over-expression, and in these clones Yorkie may be localised to the nucleus, showing inactivation of the Hippo pathway (Menendez et al., 2010). More convincingly, over-expression of wild-type Yorkie, which does not normally induce a phenotype (Dong et al., 2007), suppresses apoptosis in *Lgl* mutant clones and allows them to proliferate and dominate the tissue (Menendez et al., 2010). Various components of the Hippo pathway have also been identified in a genetic screen to identify suppressors of *Minute* induced cell competition. Mutation of *warts* or *salvador* reduces suppression of Yorkie, and these mutants were able to increase survival in *Minute* cells (Tyler et al., 2007). These data support a role for the Hippo pathway in cell competition. Interestingly, scribble has been shown to negatively regulate the Hippo pathway in zebrafish and mammalian cells. Over-expression of scribble in mammalian cell culture inhibits transcription from a YAP1 reporter (YAP1 is the mammalian homologue of Yorkie) (Skouloudaki et al., 2009). In zebrafish embryos, knockdown of scribble causes a modest increase in YAP1 target gene transcription, and acts synergistically with Fat knockdown (a known repressor of YAP1 activity) to give a large increase in YAP1 target gene transcription (Skouloudaki et al., 2009). With these data in mind, it is important to address the role of Hippo signalling in outcompeted *scribble* mutant clones in *D. melanogaster*.

In a significant breakthrough in cell competition, Rhiner et al have uncovered a signalling code that identifies cells that are outcompeted and may signal to their neighbours that they should be removed. Using a microarray, Rhiner et al

identified several genes that were specifically activated in cells with low levels of dMyc expression relative to their neighbours (Rhiner et al., 2010). The most interesting of these transcripts that they found only in outcompeted cells was called Flower (Fwe). There are 3 splice variants of Fwe: Fwe^{ubi}, which is expressed in all imaginal disc cells, and Fwe^{Lose-A} and Fwe^{Lose-B}, which as their names suggest, are only expressed in outcompeted “loser” cells (Rhiner et al., 2010). Fwe^{Lose-A/B} are expressed in outcompeted *scribble*, *tkv*^{-/-} and *Minute* cells, showing that it is not specific for Myc induced cell competition. Interestingly, Fwe^{Lose-A/B} is expressed in all cells of outcompeted clones, not just those that are in contact with wild-type, or “winner” cells. This suggests that the interaction with wild-type cells does not specifically induce expression of Fwe^{Lose-A/B}, or alternatively that the outcompeted signal is spread from cells at the boundary of a clone to those in the interior (Rhiner et al., 2010). Expression of Fwe^{Lose-A/B} is not sufficient to induce cell death, as animals that only express this form are viable. However, contact of wild-type cells with cells over-expressing Fwe^{Lose-A/B} or mutant for Fwe^{ubi}, in imaginal discs or in a *D. melanogaster* cell line, is sufficient to induce cell death in the Fwe^{ubi} mutant or Fwe^{Lose-A/B} expressing cells. Finally, in experiments that rescue outcompeted clones such as over-expression of Rab5 or Dpp, Fwe^{Lose-A/B} is not activated or activated less frequently. Inhibition of caspases, which blocks cell competition induced death, does not inhibit expression of Fwe^{Lose-A/B} (Rhiner et al., 2010). These data represent a serious advance in the field of cell competition and place Fwe as a central regulator in the process that lies downstream of events such as a reduction in survival signalling, but upstream of caspase activation. It is particularly interesting that splice variants of the same gene are able to induce such a phenotype. Fwe localises to the cell membrane but it is not yet known what the molecular function of the Fwe is. This work raises some very interesting questions, such as how Fwe signals to activate cell death pathways. It is important to examine the conservation of Fwe across species.

1.12 Cell competition that does not result in cell death

I have so far described examples where competition between different cell types has resulted in the death of the outcompeted cells. A few examples have been described where cells can compete without cell death. One of these studies focused on the stem cell niche in the *D. melanogaster* ovary germline (Rhiner et al., 2009). In the ovary germline there are two germline stem cells that lie side by side in the stem cell niche. These divide asymmetrically to give rise to the other lineages of the ovary (reviewed in (Huynh and St Johnston, 2004)). These stem cells express dMyc; over-expression of dMyc in a stem cell results in this stem cell overtaking the entire germline, replacing the other stem cell in the process (Rhiner et al., 2009). This type of cell competition is not dependent on cell death- instead it is assumed that the outcompeted stem cell is driven to differentiation by an unknown mechanism. Similar to cell competition in imaginal discs, competition for extra-cellular Dpp seems to be important as this is a critical signalling factor in the germline niche, and stem cells that over-express dMyc have increased Dpp signalling (Rhiner et al., 2009). The cell competition inducing mutations *scribble* and *lgl*^{-/-} were tested in this system, as well as mutations of the growth regulatory Hippo and Hedgehog signalling families, and did not give a competition phenotype (Rhiner et al., 2009). This phenotype appears to be less general than cell competition in imaginal discs.

A recent study in mice has shown that competitive interactions occur during haematopoietic system re-population, under specific conditions (Bondar and Medzhitov, 2010). In an assay of bone marrow re-population after irradiation, injecting a mix of 90% WT and 10% *p53*^{+/-} bone marrow cells followed by a small, non-lethal dose of irradiation results in the *p53*^{+/-} cells expanding and making up a disproportionately large percentage of the haematopoietic stem cells and circulating blood cells (Bondar and Medzhitov, 2010). Further experiments that alter p53 gene dosage by different methods show that cells that have lower p53 activity are somehow able to outcompete cells with higher p53 activity following irradiation. Apoptosis is not required for the *p53*^{+/-} cells to dominate the haematopoietic lineage. Intriguingly, the cells with higher p53 have decreased levels of various pro-proliferation genes, and up-regulate genes that

are associated with senescence, such as p16^{INK4A} (Bondar and Medzhitov, 2010). The conclusion from this study that cells can be outcompeted into senescence rather than cell death is a very interesting one. The authors use the term cell competition to refer to the phenotype described, but the phenotype they report bears little similarity to the cell competition phenotype described in *D. melanogaster*. Most importantly, competition only occurs between the two cell types after stress induced by irradiation and the cells are not epithelial cells as in studies in *D. melanogaster* (Bondar and Medzhitov, 2010, Moreno, 2008). Consequently, there is no evidence that contact between the two populations is required for the induction of senescence, and the data may be explained by the p53 heterozygous cells being selected for, rather than actively outcompeting wild-type cells. These caveats mean that this study does not fit the description of “cell competition” as previously outlined (Moreno, 2008, Johnston, 2009). Despite these drawbacks, this remains an interesting and important study. Clarification of the mechanism by which wild type cells are pushed into senescence is important for proper appraisal of these findings.

1.12 Cell competition in mammalian cells

As is apparent by the emphasis of this introduction, the vast majority of research into cell competition has been performed in *D. melanogaster*. The analysis of mutants in clones in an otherwise wild-type tissue is a common technique in *D. melanogaster*, so this in many ways makes it the ideal organism. Nonetheless, there have been some substantial studies in mammalian cells that require discussion.

Cell culture has proved an amenable tool for analysis of cell competition, as different cell lines can easily be mixed to create mosaics of cells similar to those in *D. melanogaster*. Using this approach it has been shown that a single epithelial cell that over-expresses oncogenic RasV12 or v-Src is apically extruded from the epithelial monolayer (Hogan C et al., 2009, Kajita M et al., 2010). When cultured alone, v-Src or RasV12 expressing cells are able to form epithelial monolayers

similar to non-transformed cells. Apical extrusion is, therefore, due to the interaction of the transformed cell with non-transformed neighbours. This form of apoptosis independent cell competition is conserved across species; cells over-expressing RasV12 are apically extruded in *D. melanogaster* (Hogan C et al., 2009) and cells with increased Src activity are removed basally in *D. melanogaster* and apically extruded in *Danio rerio* (zebrafish) (Vidal et al., 2006, Kajita M et al., 2010).

Apoptosis dependent cell competition has also been reported in a mammalian cell culture system. Mahjong is an Lgl binding protein; clones of *Mahjong* mutant cells in *D. melanogaster* are removed from the epithelium by apoptosis but whole mutant flies are viable, although they develop more slowly than wild-type (Tamori et al., 2010). These findings are reproducible in mammalian epithelial cell lines; small numbers of cells that express an anti Mahjong shRNA die when they are surrounded by normal epithelial cells (Tamori et al., 2010), but knockdown of Mahjong itself does not induce cell death. Death of Mahjong deficient cells, in *D. melanogaster* or in mammalian epithelial cells, is dependent on activity of JNK (Tamori et al., 2010). This shows that the role of JNK in cell competition may be evolutionarily conserved.

There are currently very few reports of cell competition studies in mammalian model organisms. It is extremely technically demanding to produce and follow the fate of clones in mice and investigating the molecular mechanism would be more demanding still. Despite this, a handful of reports from experiments in mice suggest there is potential in investigating cell competition in mammalian model organisms. As well as the study in the mouse bone marrow by Bondar and Medzhitov already described (Bondar and Medzhitov, 2010), Oliver et al have described a murine *Minute* called Belly spot and tail (Bst) (Oliver et al., 2004). *Bst* is, like *Minute*, a homozygous lethal mutation of a ribosomal protein. Heterozygous *Bst*/+ mutants develop with several abnormalities and have a reduced capability to synthesise protein, but develop to maturity and are viable (Oliver et al., 2004, Rice et al., 1997, Rice et al., 1995). Oliver et al injected LacZ expressing WT embryonic stem (ES) cells that contain a gene for agouti coat fur

into non-agouti *Bst/+* heterozygous embryos and non-agouti wild-type embryos, allowed the embryos to develop to adulthood and studied the coat colour of the adult mice. An increase in agouti colour in the coat of the adult mice indicates presence of transferred ES cells in the adult mouse. Injection of these ES cells into *Bst/+* embryos resulted in mice with large patches of agouti fur and with large patches of LacZ positive cells in various organs. Transferral of LacZ, agouti, wild-type ES cells into wild-type embryos gave mice with very few agouti patches and very little detectable LacZ in tissues (Oliver et al., 2004). These data show that the WT ES cells were able to make up a disproportionate amount of the mouse when injected into the *Bst/+* embryo; this suggests that the WT cells have a competitive advantage over the *Bst/+* cells. There is no mention of increased apoptosis in *Bst/+* cells that neighbour WT cells in these chimeric animals. This is evidence that the principle that cells with lower ribosomal activity are outcompeted by wild-type cells, as demonstrated extensively in *D. melanogaster* with *Minute* mutants, is conserved in mammals.

Chapter 2 Materials and Methods

2.1 Molecular Biology and Biochemistry

2.1.1 Construction of pTR MDCK scribble shRNA cell line

The construct pSUPERIOR scribble shRNA was made by amplifying the following sequences by polymerase chain reaction (PCR):

5'-GATCCCCCAGATGGTCCTCAGCAAGTTTCAAGAGAACTTGCTGAGGACCATCTGT
TTTTC-3'

5'-TCGAGAAAAACAGATGGTCCTCAGCAAGTTCTCTTGAACTTGCTGAGGACCATC
TGGGG-3'

These sequences were chosen as they have been shown to achieve efficient knockdown of scribble expression in MDCK cells (Qin et al., 2005b). These oligonucleotides were ligated into pSUPERIOR-neo-GFP (Oligoengine) between the BglII and XhoI sites. The pSUPERIOR range of constructs allows stable expression of short interfering RNA in mammalian cells (Brummelkamp et al., 2002). Inducible expression of scribble shRNA was achieved using the Tet-ON system (Invitrogen). pSUPERIOR scribble shRNA was then stably transfected into MDCK cells that are stably transfected with the construct pcDNA6/TR (Invitrogen) (pTR MDCK) using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen). Stably transfected cells were selected by addition of the antibiotic neomycin to the culture medium. Clones of MDCK cells containing pcDNA6/TR and pSUPERIOR-neo-GFP were then screened for knockdown of scribble when incubated with tetracycline (10µg/ml) and the clone with the most efficient knockdown was used in experiments.

2.1.2 Construction of pTR MDCK scribble shRNA hScrib cell line

A construct containing the cDNA of human scribble was kindly donated by M. Sebbagh (pECFP-C1 hScrib). hScrib cDNA was excised from this construct and ligated into between the XbaI and BamHI sites of pcDNA4/TO (Invitrogen). pTR MDCK scribble shRNA cells were then stably transfected with pcDNA4/TO hScrib using Lipofectamine 2000. Stably transfected cells were selected by zeocin

resistance conferred by the pcDAN4/TO hScrib construct. Expression of hScrib was induced by addition of tetracycline (10 µg/ml) and a suitable clone was identified and used in experiments.

2.1.3 SDS sample preparation

Culture medium was aspirated and cells were washed once in cold PBS. 1 ml PBS was added and the cells scraped from the dish using a cell scraper and pipetted into a 1.5ml tube. Samples were then centrifuged at 4000 RPM for 3 minutes to pellet the cells. The supernatant was removed and cells were lysed in lysis buffer (20mM Tris/Cl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100 (all Sigma, MO)) supplemented with 5 µg/ml leupeptin (Sigma), 50 mM phenyl methyl sulphonyl fluoride (PMSF, Sigma) and 7.2 trypsin inhibitor units/ml aprotinin (Sigma). When blotting for phosphorylated proteins 10 mM NaF, 0.1mM sodium orthovanadate and 0.1 mM ammonium molybdate (all Sigma) were also added to inhibit phosphatases. Samples were rotated for 30 minutes at 4°C. 100µl of the sample was mixed with 50µl SDS buffer and incubated at 95°C for 10 minutes.

2.1.4 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gels were made as described in the table below. N, N, N, N-tetramethyl-ethylenediamine (Fluka) and ammonium persulphate (APS) (Sigma) were added last as they induce acrylamide polymerisation.

Table 3 Composition of SDS-PAGE gels used in this thesis

Resolving gel	8%		10%	
	<i>mini</i>	<i>maxi</i>	<i>mini</i>	<i>maxi</i>
Water	3.5ml	14ml	3ml	12ml
1.5M Tris/Cl (pH 8.8)	2.5ml	10ml	2.5ml	10ml
50% glycerol	2ml	8ml	2ml	8ml
40% acrylamide	2ml	8ml	2.5ml	10ml
10% SDS	100µl	400µl	100µl	400µl
TEMED	15µl	50µl	15µl	50µl
10% APS	30µl	120µl	30µl	120µl

Stacking gel	mini	maxi
Water	3.2ml	9.5ml
0.5M Tris/Cl (pH 6.8)	1.25ml	3.75ml
40% acrylamide	500µl	1.5ml
10% SDS	50µl	150µl
TEMED	5µl	15µl
10% APS	50µl	150µl

Gels are loaded into a Bio-Rad Mini Protean II gel electrophoresis tank and the inner tank filled with Laemmli buffer (National Diagnostics) until overflowing and covering the bottom of the gels. Typically, 30µl of the desired SDS sample was added to a well and Colorburst Markers (Sigma) were used as protein markers. Gels were run at 15mA (1 gel) or 30mA (2 gels) for 2-3 hours, depending on the protein studied.

2.1.5 Western blotting

Western blotting was performed using Bio-Rad Mini Protean II equipment. Polyvinylidene fluoride membrane (PVDF, Millipore) was soaked in methanol and then shaken in transfer buffer (100 mM glycine, 10 mM Trizma base, 10% methanol (all Sigma)) until all drops of methanol were washed off. Small sheets of Whattman blotting paper and sponges were also soaked in transfer buffer. A transfer cassette was prepared as follows: a sponge was placed on the black side, then 3 sheets of pre-soaked blotting paper followed by the polyacrylamide gel. The pre-prepared PVDF membrane was then placed upon the gel with a cut on the top left corner to orient the membrane post transfer. Three sheets of blotting paper were put on the membrane, rolling with a plastic pipette after each sheet to remove air bubbles. Finally a second sponge was put on top of the blotting paper and the cassette closer and placed in the transfer tank and the tank filled with transfer buffer. Transfers were run at 50 V for 90 minutes at 4°C or 25 V overnight at 4°C.

Membranes were blocked in 3% Marvel milk powder/PBS for 1 hour at room temperature, primary antibodies were incubated in 3% milk/PBS for 2 hours at room temperature or overnight at 4°C. Membranes were washed at least three times for 10 minutes in PBS-Tween (0.05% v/v Tween 20). When blotting for phosphorylated proteins, membranes were blocked and antibodies diluted in 5% w/v bovine serum albumen (BSA) (Sigma) and 0.1% Tween 20 (Sigma) in Tris-buffered saline (TBS)(50 mM Tris/Cl pH 7.4, 150 mM NaCl). HRP-conjugated antibodies were incubated at room temperature for 2 h. Bands were visualised using ECL western blotting detection reagent (Amersham, UK) and high performance chemiluminescence film (Hyperfilm, Kodak) according to manufacturer's instructions.

2.1.6 RNA Extraction and Reverse transcription

Before isolation of RNA from cells, pipettes and work surfaces were treated with RNase Zap (Sigma). Filter pipette tips were used to prevent contamination with RNases. RNA was isolated from cells using RNeasy Mini Kits (Qiagen). cDNA was made from RNA using the Superscript III First-Strand kit (Invitrogen)

2.1.7 qPCR

A Realplex² Mastercycler qPCR machine (Eppendorf) was used to quantitative polymerase chain reaction experiments. Each reaction contained 300 ng/μl DNA, 250 μM forward primers, 250 μM reverse primers and 12.5 μl Dynamo Flast Master Mix (Finnzymes, Finland) (total reaction volume was 25 μl). Thrombospondin 1 primers were from Qiagen. Actin primer sequences were: forwards 5'-GCGAGAAGATGACCCAGAT-3' and reverse 5'-TGGTGGTGAAGCTGTAGCC-3'.

2.1.8 Screening of conditioned culture medium

Cells were seeded in four different conditions at 10×10^6 per 15 cm culture dish, with each condition seeded in triplicate. Tetracycline was added for 48 h. For the final 24 h, culture medium was changed to serum free DMEM. Medium was

collected and centrifuged at 4000 RPM for 5 mins at 4 °C to pellet dead cells. Centrifuged medium was then pushed through a 0.45 µm filter to remove cell debris that would block future filtration. 15 ml filtered medium was added to a Amicon Ultra 15 Ultracell 10K filtration unit (Millipore, MA). These filters are able to retain protein but water passes through the filter. The filter unit was centrifuged at 14000 rpm for 30 mins at 4 °C. Filtrate was removed and more conditioned medium was added to the filter unit and the unit was centrifuged as before. This was repeated until the conditioned medium was concentrated to approximately 200 µl. This was then removed from the filter unit using a P200 pipette. 100 µl SDS buffer was added and heated for 5 mins at 95 °C.

Boiled conditioned medium was then run on a 8% SDS-PAGE gel using as described. Gels were stained and visualised using SYPRO Ruby Protein stain to visualise protein according to manufacturer's instructions (Invitrogen).

2.2 Cell Biology

2.2.1 Cell Culture

Madin-Darby Canine Kidney (MDCK) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with foetal calf serum (Sigma-Aldrich, to a final concentration of 10%), Glutamax™ (Gibco, 1%) and penicillin/streptomycin (PAA Laboratories GmbH, 1%). MDCK pTR Scribble shRNA cells were grown in DMEM supplemented with tetracycline free foetal bovine serum (to a final concentration of 10%), Glutamax™ (Gibco, 1%), penicillin/streptomycin (Gibco, 1%), G418 sulphate (Calbiochem, 800µg/ml) and 5µg/ml blasticidin (PAA) to maintain selection for the constructs pSUPERIOR scribble shRNA and pcDNA6/TR (Invitrogen). Mixed MDCK/MDCK pTR Scribble shRNA cultures were grown in DMEM containing 10% tetracycline free foetal bovine serum, 1% Glutamax™ and 1% penicillin/streptomycin (referred to as MDCK Mix). A stock of tetracycline (Sigma Aldrich) was diluted in ethanol and stored at -20°C. When required, tetracycline was added to cells at a final concentration of 10µg/ml. All cells were grown at 37 °C with 5 % CO₂.

2.2.2 Passage of cells

Cells were grown to approximately 80% confluence before splitting, in order to prevent overcrowding and loss of epithelial morphology. Cell culture medium was aspirated and cells were washed once with sterile PBS before addition of Trypsin/EDTA solution (LMCB stores) for 5-10 minutes, until cells became detached from the culture dish and each other. Fresh medium was added to the trypsinised cells and they were seeded into fresh plates with fresh medium.

When cells were split to setup mixed culture experiments, trypsinised pTR MDCK scribble shRNA cells were pipetted up and down 3 times in order to break apart any clumps of cells. A suspension of single cells was then mixed with normal MDCK cells and plated.

2.2.3 Freezing/thawing of cells

To freeze cells for long-term storage, cells were trypsinised as described above. Instead of re-plating, cells were re-suspended in fresh medium and pipetted into a 15 ml tube (BD Biosciences, NJ) and centrifuged for 5 minutes at 800 RPM. Supernatant was aspirated and cells were re-suspended in MDCK Mix containing 10% dimethyl sulphoxide (DMSO, Sigma Aldrich). Cells were then aliquoted into Cryotubes and frozen to -80 °C for 2 days prior to transfer to liquid nitrogen.

To thaw frozen cells, the cryotube was removed from liquid nitrogen and thawed in by warming in hands. Cells were removed into a 15 ml tube and centrifuged for 5 minutes at 800 RPM. Cells were re-suspended in fresh medium and plated into a culture dish. Medium was changed to the appropriate selective medium the day after thawing.

2.2.4 Fluorescent labelling of cells

Green fluorescent CMFDA and red fluorescent CMTPX dyes were used to fluorescently label cells according to manufacturer's instructions (Invitrogen).

2.2.5 Time-lapse Microscopy

Cells were seeded in 6 well glass bottomed dishes (MatTek Corporation, MA, USA) at a density of 3.5×10^5 cells per well. Mixed cultures were created by mixing 3.5×10^5 MDCK cells with 1.75×10^4 scribble shRNA cells (to make a 1:10 dilution) in a 15 ml tube prior to seeding. When applicable, tetracycline was added 6 hours after seeding at a final concentration of 10 $\mu\text{g/ml}$. Prior to imaging, the culture medium was changed to Leibovitz's L-15 medium (Gibco) supplemented in the same way as DMEM medium. Fresh tetracycline was added and when desired small molecule inhibitors were added. Sytox Blue (Invitrogen) was added at a dilution of 1:5000. Imaging was begun 24 hours after the addition of tetracycline and proceeded for an additional 40 hours. Images were acquired with a Hamamatsu Retiga Exi camera at 20x magnification every 10 minutes. Fluorescence images were acquired every 6 time-points. Images were acquired and analysed using Volocity 5.3 software (Perkin Elmer, MA). Percentage of dead cells was measured by dividing the number of stained cells that died during live-imaging by the total number of stained cells at the end of the experiment, alive and dead.

2.2.6 Culture of MDCK cells in three-dimensional cysts

MDCK cells were trypsinised and re-suspended to a concentration of 1×10^6 cells/ml in DMEM (free of supplements). To make 1 ml of gel 2.5×10^5 cells were suspended in 290 μl DMEM. 290 μl DMEM (10% FCS), 20 mM HEPES/NaOH pH 7.4 and 400 μl 5mg/ml rat type I collagen (R & D Systems) were then added sequentially. The suspension was gently mixed by pipetting and 100 μl was added to each well of a 8-well Chamberslide (BD Biosciences). The slide was placed in 37°C 5% CO₂ incubator to solidify the gel before 450 μl culture medium was added to each well, along with tetracycline when desired. Culture medium was replaced every two days and cells were fixed and analysed after 13 days.

To prepare for immuno-staining, wells were washed three times with PBS followed by incubation with 100 U collagenase VII (Sigma) diluted in PBS for 15

mins. Collagenase was removed and wells were incubated with 4% paraformaldehyde diluted in PBS (PFA) for 30 mins followed by permeabilisation with 0.5% Triton X-100/PBS. Wells were washed once in PBS and then washed 3 times (10 minutes each wash) in glycine wash buffer (130 mM NaCl, 7 mM Na₂HPO₄, 4 mM NaH₂PO₄, 100 mM glycine) followed by blocking in cyst block buffer for more than 1 hour (130 mM NaCl, 7 mM Na₂HPO₄, 4 mM NaH₂PO₄, 7.6 mM NaN₃, 0.02% w/v BSA (Sigma), 0.5% Triton X-100, 0.05% Tween20, 10% FCS). After blocking, wells were incubated with primary antibodies in 200 µl cyst block buffer overnight at 4°C. Primary antibody was removed and wells were washed three times (20 mins each) in cyst block buffer before incubation of secondary antibodies and/or phalloidin in 200 µl cyst block buffer for 3 h at room temperature. Secondary antibodies were removed, wells washed three times in cyst block buffer (20 mins each), once in PBS and post-fixed in 4% PFA for 15 mins. Wells were then incubated with Hoechst 33342 to visualise DNA and washed twice in PBS. The Chamberslide was disassembled and gels were mounted using Mowiol (33.3% glycerol (Sigma), 13.3% Mowiol (Calbiochem) in 0.13 M Tris/Cl pH 8.5) and covered with a glass coverslip. Cysts were studied using a Leica SPE confocal microscope and Leica LAF imaging software. Confocal data was coloured using ImageJ (NIH, USA) and merges created and linearly manipulated using Adobe Photoshop CS4 (Adobe, CA).

2.2.7 Preparation of collagen-coated coverslips

Collagen matrix was prepared by mixing 7 ml collagen (NITTA Gelatin, Japan) with 2 ml 5 X DMEM (Gibco) and 1 ml reconstitution buffer (0.05 N NaOH (VWR), 200 mM HEPES (Gibco) and 260 mM NaHCO₃ (Sigma)) on ice. 500 µl of collagen mix was then pipetted onto glass coverslips in 12 well plates and incubated at 37°C for 30 minutes. After this incubation cover-slips are ready for seeding with cells.

2.2.8 Immunofluorescence

Cells were seeded onto collagen-coated coverslips at a density of 8×10^4 cells per well of a 12 well plate. Cells were washed twice with PBS and fixed in 4% PFA at

37°C for 15 minutes. Post fixing the cells were washed once with PBS and incubated with 0.5% Triton X 100/PBS for 15 minutes at room temperature. Cells were then washed twice with TBS for 2 minutes and blocked in cyst blocking buffer (20mM Tris/Cl pH 7.4, 100 mM NaCl, 7.68 mM sodium azide, 0.2% Triton X 100, 0.05% Tween 20 (Sigma), 1% BSA) for at least 2 hours. After blocking the cover-slips were removed from the 12-well plate using a scalpel and placed on Parafilm (Pechiney Plastic, IL). Primary antibodies were diluted in cyst blocking buffer and 60 µl was added to each cover-slip and incubated overnight at 4°C. Cover-slips were washed in TBS 3 times. Secondary antibodies and phalloidin (when required) were incubated in cyst block buffer for 2 hours at room temperature. Cover-slips were then washed 3 times in TBS and incubated with Hoechst 33342 (pre-diluted 1:5000 in PBS) for 2 minutes to visualise DNA (referred to as DAPI in figures for simplicity). Cover-slips were then mounted onto slides, collagen facing up, covered with 200 µl Mowiol mounting solution and a 52 mm x 20 mm glass cover-slip was gently placed on top. Slides were allowed to dry in the dark before being stored at 4°C. Images were acquired using a Leica SPE confocal microscope and Leica LAF imaging software. Confocal data was coloured using ImageJ, merges created and levels linearly manipulated using Photoshop.

2.2.9 Phase contract microscopy

Cells were seeded at a concentration of 1×10^5 cell/well of a 6-well plate. Tetracycline was added for 72 h where appropriate. Phase contrast images were acquired using a Leica DMIRB microscope and OpenLab (Perkin Elmer, MA). Images were cropped using Adobe Photoshop.

2.2.10 Methanol fixation

Cells were seeded onto glass coverslips at a density of 2×10^5 cells per well of a 6-well plate. Cells were washed in PBS twice and 1 ml of ice-cold methanol (Sigma) was added to the coverslips and coverslips were incubated at -20°C for 5 minutes. Cells were washed twice in PBS and incubated with antibodies as described above.

2.2.11 BrdU labelling

Cells were trypsinised and seeded onto glass cover-slips at a density of 2×10^5 cells per well of a 6-well culture plate. Where appropriate, cells were incubated with tetracycline for a total of 72 h. For the final 6 h of incubation, medium on all cells was changed to FCS free DMEM and bromodeoxyuridine (BrdU) was added according to manufacturer's instructions (Calbiochem). Cells were washed twice with PBS and fixed in 4% PFA for 15 mins before permeabilisation in 0.5% Triton X 100/2 M HCl for 30 minutes. Cells were washed twice in PBS and blocked with 3% BSA/PBS for 15 minutes. Anti-BrdU antibody (mouse, Calbiochem) was diluted 1:100 in 0.1% BSA/PBS, added to cover-slips and incubated for 1 h. Cells were washed six times in PBS before incubation with anti-mouse AlexaFluor 568 secondary antibody diluted 1:200 in 0.1% BSA. Cells were washed six times in PBS before mounting in Mowiol. Cells were analysed using a Leica microscope. At least 200 cells in were analysed for each condition in three independent experiments.

2.2.12 Antibodies

A list of antibodies used in this these. Where a protein is studied by Western blotting (WB) and immunofluorescence (IF) the antibody used for each application is indicated.

Primary antibodies

Table 4 A list of primary antibodies used in this thesis

Target	Dilution (1:)	Species	Source
Cleaved caspase-3	100	Rabbit	Cell Signalling (MA)
p38MAPK	100	Rabbit	Cell Signalling
pT180/Y182	100	Rabbit	Abcam (UK)
JNK 1 + 2	100	Rabbit	Abcam (UK)

pT183/Y185			
Active N-Bak	100	Mouse	Calbiochem
Active N-Bax	50	Mouse	Sigma
E-cadherin (WB)	1000	Mouse	BD Biosciences (NJ)
E-cadherin ECCD2 (IF)	200	Rat	Zymed (CA)
SMAD2 pS465/467	1000	Rabbit	Cell Signalling
SMAD4	100	Mouse	Santa Cruz (CA)
ZO-1	100	Rabbit	Zymed
Tubulin	1000	Mouse	Sigma
GAPDH	5000	Mouse	Millipore
Scribble (WB)	1000	Goat	Santa Cruz
Scribble (IF)	100	Goat	Santa Cruz
Beta-catenin (WB)	1000	Mouse	BD Biosciences
Beta-catenin (IF)	100	Mouse	BD Biosciences
			G. K. Ojakian (SUNY Downstate Medical Center, NY)
gp135	100	Mouse	
pMAD	500	Rabbit	F. Pichaud (MRC LMCB)
Thrombospondin 1	1000	Mouse	Sigma
			T. Volk (Weizmann Institute, Israel)
TSP	100	Rat	
p120 catenin	100	Mouse	BD Biosciences

Secondary antibodies

Table 5 A list of secondary antibodies used in this thesis

Target	Dilution (1:)	Species	Source
Phalloidin 647	400	N/A	Invitrogen
Phalloidin 568	200	N/A	Invitrogen
AlexaFluor Anti-Rat 568	200	Goat	Invitrogen
AlexaFluor Anti-Rabbit 568	200	Donkey	Invitrogen
AlexaFluor Anti-Goat 568	200	Donkey	Invitrogen
AlexaFluor Anti-Mouse 568	200	Donkey	Invitrogen
AlexaFluor Anti-Rabbit 647	200	Donkey	Invitrogen
AlexaFluor Anti-Mouse 647	200	Goat	Invitrogen
Anti-Mouse HRP	2000	Goat	Jackson

Anti-Rat HRP	1000	Goat	ImmunoResearch (PA) Jackson
Anti-Goat HRP	1000	Mouse	ImmunoResearch (PA) Sigma
Anti-Rabbit HRP	5000	Goat	Jackson ImmunoResearch (PA)

2.2.13 Inhibitors

Chemical inhibitors used in this study are as below:

Table 6 A list of inhibitor used in this thesis

Chemical	Target	Concentration	Source
Z-VAD-FMK	caspases	100 μ M	Calbiochem
NSC23766	Rac1	100 μ M	Tocris (UK)
SB202190	p38MAPK	10 μ M	Calbiochem
SP600125	JNK1 ,2, 3	5 μ M	Sigma
SD 208	TGF- β receptor 1	2 μ M	Tocris Toronto Research Chemicals (Canada)
Blebbistatin	Myosin II	50 μ M	

2.3 Drosophila methods

2.3.1 Strains and crosses

Flies containing UAS-TSP (on the third chromosome) were kindly provided by Talila Volk (Weizmann Institute of Science, Israel). These were crossed with the line *hsflp;;tub>LacZ>Gal4*. Larvae from this cross were heat-shocked for 30 mins at 37 °C to induce expression of UAS-TSP in a mosaic manner. Third instar larvae were dissected 48 h after heat-shock and processed for immunofluorescence.

The line carrying the scribble mutant allele was *w;;FRT82B scrib¹/TM6 Tb*. *Scrib¹* is a mutation of leucine 223 to glutamine and is a genetic null mutation (Bilder and Perrimon, 2000, Zeitler et al., 2004). This line was crossed with *hsflp;;FRT82B ubi GFP/TM6*. Non-TM6 larvae from this cross were heat-shocked

for 30 mins at 37 °C to induce twin-spot clones of cells homozygous for *scrib* mutation (no GFP) and a twin spot of wild-type cells (GFP/GFP). Larvae from this cross were dissected 48 h after heat shock.

All stocks were kept at 25 °C except when being heat-shocked. Strains were kindly donated by members of the Pichaud lab unless otherwise stated.

2.3.2 Immuno-staining

Larvae were dissected in PBS and fixed in 4% PFA for 15 mins, followed by three washes in 0.05% Triton X 100/PBS (PBT) and permeabilisation in 0.5% Triton X 100/PBS. Dissected larvae were blocked in 4% BSA/0.1% Triton X 100/PBS (block buffer) for 2 h. Primary antibodies were incubated in block buffer with gentle agitation for 2 h at room temperature or 4 °C overnight. Antibody was removed and dissected larvae were washed three times (10 mins) in PBT before incubation of secondary antibodies as described for primary antibodies. Antibody was removed, dissected larvae were washed three times (10 mins) in PBT, incubated with Hoechst 33342 for 15 mins at room temperature before a final wash in PBT and mounting of imaginal wing-discs in VectaShield (Vector Labs, CA) covered by a glass cover-slip. Discs were analysed with confocal microscopy as described above.

2.4 Data analysis

2.4.1 Measurement of immuno-fluorescence intensity

Images were acquired with identical settings using Leica LAF software. Fluorescence intensity was measured using Metamorph software (Molecular Devices, CA). For analysis of p38MAPK intensity, fluorescence intensity was measured at eight 49 pixel² circles randomly assigned to the cytosolic area of each cell. The mean of these data points from more than 20 different cells was used for statistical analysis. For E-cadherin intensity analysis, 49 pixel² circles were placed at cell-cell contact regions of cells. At least 20 cell-cell contacts were

measured for each condition in each experiment. The mean of all these points was used in statistical analysis.

2.4.2 Measurement of cell height

Images were acquired with identical settings using confocal microscopy. Phalloidin was used to visualise the outline of cells. At least 20 cells were counted for each condition in three independent experiments using Metamorph software.

2.4.3 Statistics

All statistical analyses were performed using Microsoft Excel (Microsoft, WA). One tailed, non-equal variance Student's T-tests were used.

Chapter 3. Effect of scribble knockdown on MDCK cell morphology, polarity and proliferation

Chapter 3: Effect of scribble knockdown on MDCK cell morphology, polarity and proliferation

3.1 Introduction

In order to address the question of the fate of small numbers of scribble-knockdown epithelial cells surrounded by normal epithelial cells it is essential to make a cell line stably expressing an inducible anti-scribble shRNA. It is important that expression of the anti-scribble shRNA is inducible because this means mixed cultures can be made that have proper cell-cell contacts prior to knockdown of scribble. This is a more physiological reproduction of early tumourigenesis than the combination of normal epithelial cells with cells with scribble already knocked down. To this end, this chapter describes the construction and characterisation of pTR MDCK scribble shRNA cells, a cell line that expresses an anti-scribble shRNA only when tetracycline is present in the culture medium.

The polarity, morphology and proliferation of this cell line, cultured with and without tetracycline, is described in this chapter. Apicobasal polarity was studied because a key cellular role for scribble is the regulation of polarity and lack of polarity could be important when scribble-knockdown cells are surrounded by normal cells. It is described that pTR MDCK scribble shRNA cells grown without tetracycline exhibit a similar morphology to MDCK cells and that addition of tetracycline to knockdown scribble makes cells flatter and wider, but most cell-cell contact proteins are correctly localised. The effect of scribble-knockdown on 3 dimensional cyst formation is also studied, and it is shown that there are mild defects in this when scribble is knocked down.

Cell proliferation may be an important factor in the interaction of scribble-knockdown MDCK cells with normal MDCK cells as early descriptions of cell competition, and some contemporary ones, describe it as the elimination of slow growing cells (Morata and Ripoll, 1975, Menendez et al., 2010). Using BrdU

incorporation it is shown that knockdown of scribble does not significantly affect proliferation when compared to normal MDCK cells.

3.2.1 Construction of pTR MDCK scribble shRNA cell line

In the absence of tetracycline, expression of the anti-scribble shRNA in pTR MDCK scribble shRNA cells is repressed by the tet repressor (Hillen and Berens, 1994). Addition of tetracycline enables transcription of the anti scribble shRNA. Western blotting with an anti-scribble antibody was performed to analyse the temporal dynamics of the knockdown of scribble in pTR MDCK scribble shRNA cells. In the absence of tetracycline, the pTR MDCK scribble shRNA cell line expresses scribble at a level comparable to that seen in normal MDCK cells (Figure 5.). Addition of tetracycline reduces scribble protein level to 45% of control (pTR MDCK scribble shRNA cells in the absence of tetracycline) after 24 hours and to 9% of control after 48 hours. Scribble protein is barely detectable in pTR MDCK scribble shRNA cells incubated with tetracycline for 72 hours (Figure 5). To ensure that the protein level of critical cell adhesion molecules was also not affected by the anti-scribble shRNA, the level of E-cadherin and β -catenin was checked by Western blotting. Expression of the anti-scribble shRNA did not significantly affect the expression level of either E-cadherin or β -catenin (Figure 5). Therefore, addition of tetracycline to pTR MDCK scribble shRNA cells efficiently and specifically knocks down the level of scribble protein.

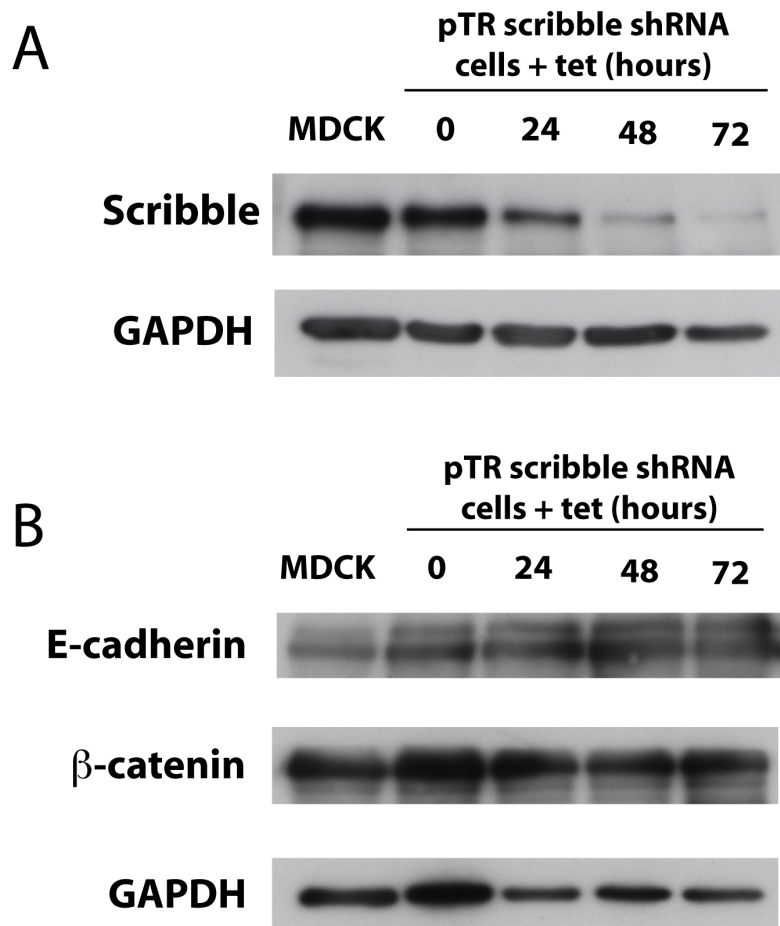


Figure 5 Addition of tetracycline reduces scribble protein level. pTR MDCK scribble shRNA cells were incubated with tetracycline for 0, 24, 48 and 72 hours. MDCK cells were also incubated with tetracycline for 72 h to control for any effects of tetracycline on protein level. SDS samples were taken and run on SDS-PAGE gels. Western blotting was performed with antibodies against scribble (A), E-cadherin and β -catenin (B). GAPDH was used as a loading control.

3.2.2 Effect of scribble shRNA on cell morphology

Scribble is known to regulate polarity and cell morphology in *D. melanogaster* (Bilder D., 2003, Bilder et al., 2000b) and mammalian epithelial cells (Zhan et al., 2008, Qin et al., 2005b). To check the effect of scribble-knockdown on the morphology of MDCK cells, tetracycline was added to pTR MDCK scribble shRNA cells for 72h and the morphology of live cells was studied with phase contrast microscopy. Normal MDCK cells and pTR MDCK scribble shRNA cells cultured without tetracycline exhibit a typical epithelial cobblestone morphology,

grouping together in tight islands of cells (Figure 6). pTR MDCK scribble shRNA cells incubated with tetracycline for 72 h, a time point at which these cells contain very little scribble protein (5), no longer have an epithelial morphology. They appear larger and flatter than normal MDCK cells and cells that are on the edge of the colony produce large protrusions into the free space. Such protrusions cannot be seen in either MDCK cells or pTR MDCK scribble shRNA cells cultured without tetracycline. Cells are still in contact with each other so it is assumed some kind of cell-cell adhesions remain (Figure 6). No signs of migration or scattering were seen.

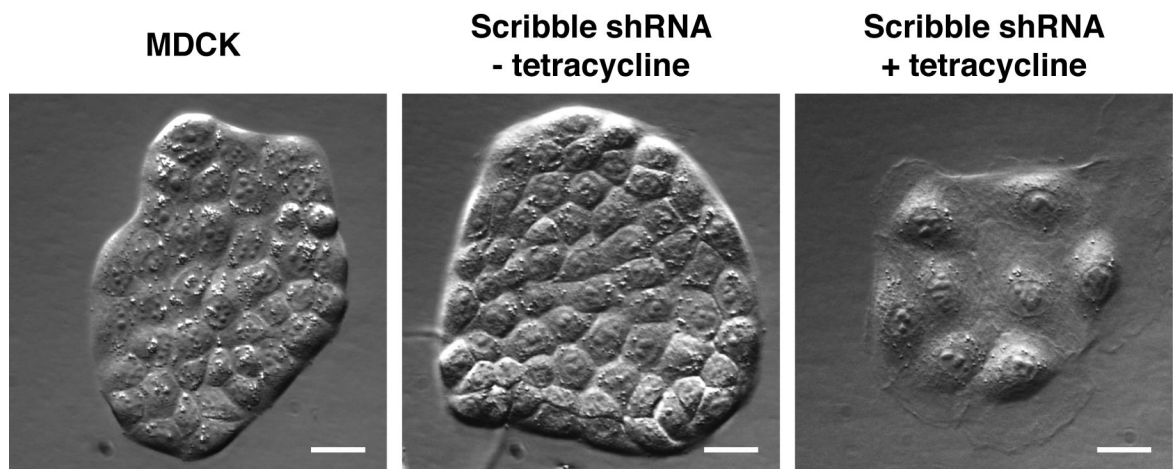


Figure 6 Effect of scribble knockdown on epithelial cell morphology. Phase contrast images of MDCK cells and pTR MDCK scribble shRNA cells. MDCK cells (left panel) and scribble shRNA cells + tetracycline (right panel) were incubated with tetracycline for 72h. pTR MDCK scribble shRNA cells lose epithelial morphology when incubated with tetracycline. Scale bars are 15 μ m.

3.2.3 Effect of scribble knockdown on cell polarity in 2D

From phase contrast images (Figure 6) it is apparent that scribble-knockdown cells have disrupted epithelial morphology. To examine the polarity and epithelial nature of scribble-knockdown MDCK cells, the distribution of proteins crucial for epithelia morphology was checked with immunofluorescence. In order to provide a more physiological growth condition, cells were cultured on coverslips that were coated in collagen, a major component of the extracellular matrix upon which epithelial cells normally grow (Bosman and Stamenkovic,

2003). MDCK cells grown on collagen matrix also polarise more fully, with a greater apical-basal cell height that makes the polarised distribution of proteins easier to study. pTR MDCK scribble shRNA cells were grown on collagen-coated coverslips for 64h +/- tetracycline and processed for immunofluorescence. The scribble complex localises to the basolateral membrane, and part of its function is to correctly localise the apical Par complex to the apical membrane (Humbert et al., 2006). To see if the apical membrane was correctly localised in scribble-knockdown MDCK cells, the localisation of the apical membrane marker gp135 was studied (Meder et al., 2005). In pTR MDCK scribble shRNA cells incubated without tetracycline, gp135 localised exclusively to the apical membrane. The same pattern of staining was seen in scribble-knockdown MDCK cells, suggesting that there is no extension of the apical membrane when scribble is knocked down (Figure 8).

Previous reports have described an intimate relationship between E-cadherin and scribble, and that knockdown of scribble in MDCK cells leads to a disruption of E-cadherin localisation at cell-cell contacts (Navarro et al., 2005, Qin et al., 2005b). Consistent with these reports, a disruption of E-cadherin localisation is seen in scribble-knockdown MDCK cells. In normal MDCK cells (in this experiment, pTR MDCK scribble shRNA cells incubated without tetracycline), E-cadherin localises at cell-cell contacts in a tight, discreet line (Figure 7, left hand panels). In scribble-knockdown cells MDCK, E-cadherin localises to cell-cell contacts but is frequently disrupted, appearing as a broad smear rather than a tight line (Figure 7, right hand panels). Also, intra-cellular staining of E-cadherin could be seen. Analysis of XZ sections through the epithelial monolayer shows that in control cells E-cadherin is mostly localised to the basolateral membrane (cell-cell contact area) with a little staining at the apical surface and none on the basal surface (Figure 8, left hand panels). In XZ sections of scribble-knockdown cells the localisation of E-cadherin was markedly disrupted (Figure 8, right hand panels). E-cadherin is present at cell-cell contacts, but E-cadherin is predominantly localised to the basal membrane of the cell. There is very little staining at the apical surface. This mislocalisation of E-cadherin could explain the lack of epithelial morphology seen in scribble-knockdown MDCK cells (Figure 6).

Studying XZ images of scribble-knockdown cells, it is also obvious that there is a significant reduction in apical-basal cell height in scribble-knockdown cells. This agrees with the flattened morphology of scribble-knockdown cells seen by phase-contrast microscopy (Figure 6).

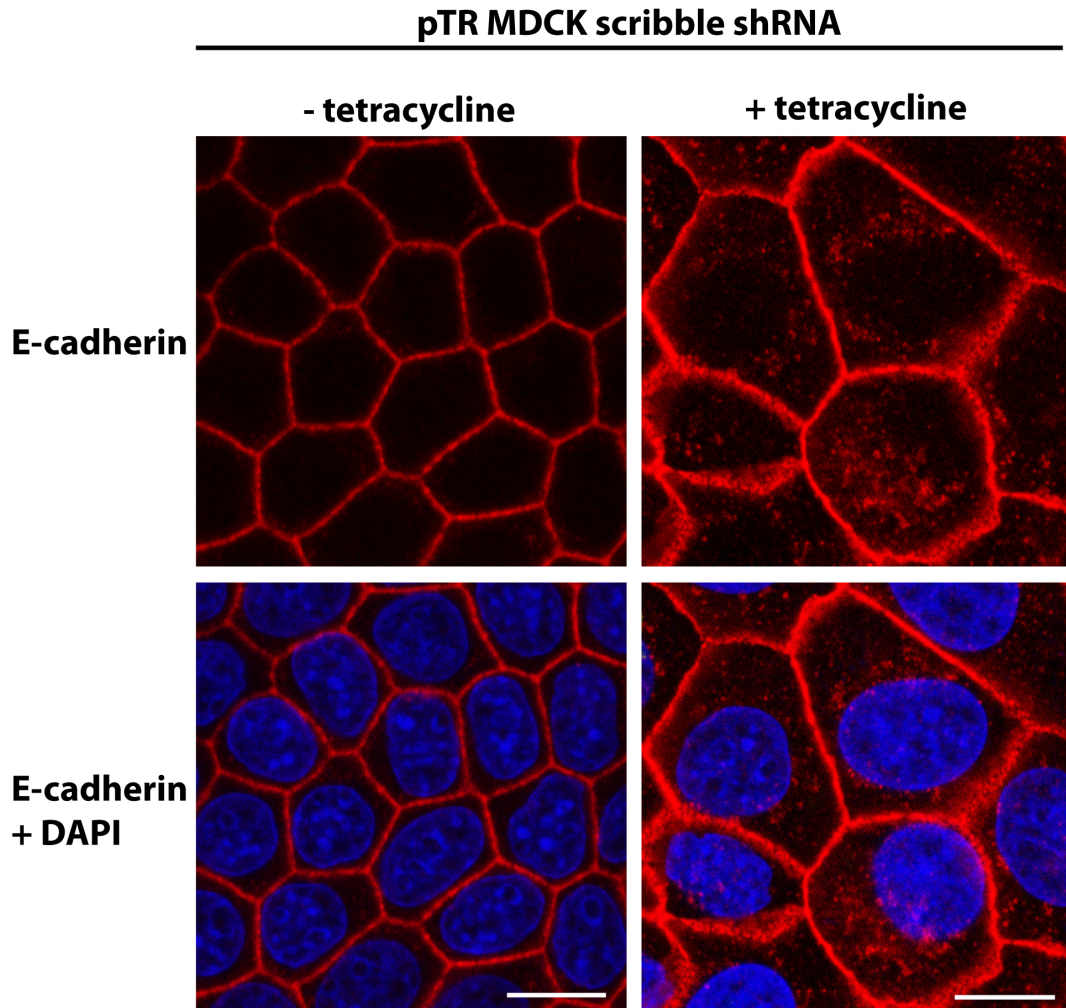


Figure 7 *Scribble knockdown causes a defect in junctional localisation of E-cadherin.* pTR MDCK scribble shRNA cells were seeded onto collagen coated coverslips and incubated +/- tetracycline for 64h before fixation and immunostaining with an anti-E-cadherin antibody. E-cadherin at cell-cell contacts in scribble-knockdown cells is frequently a wide smear rather than the tight, discreet line seen in controls. Scale bars are 10 μ m.

pTR MDCK scribble shRNA

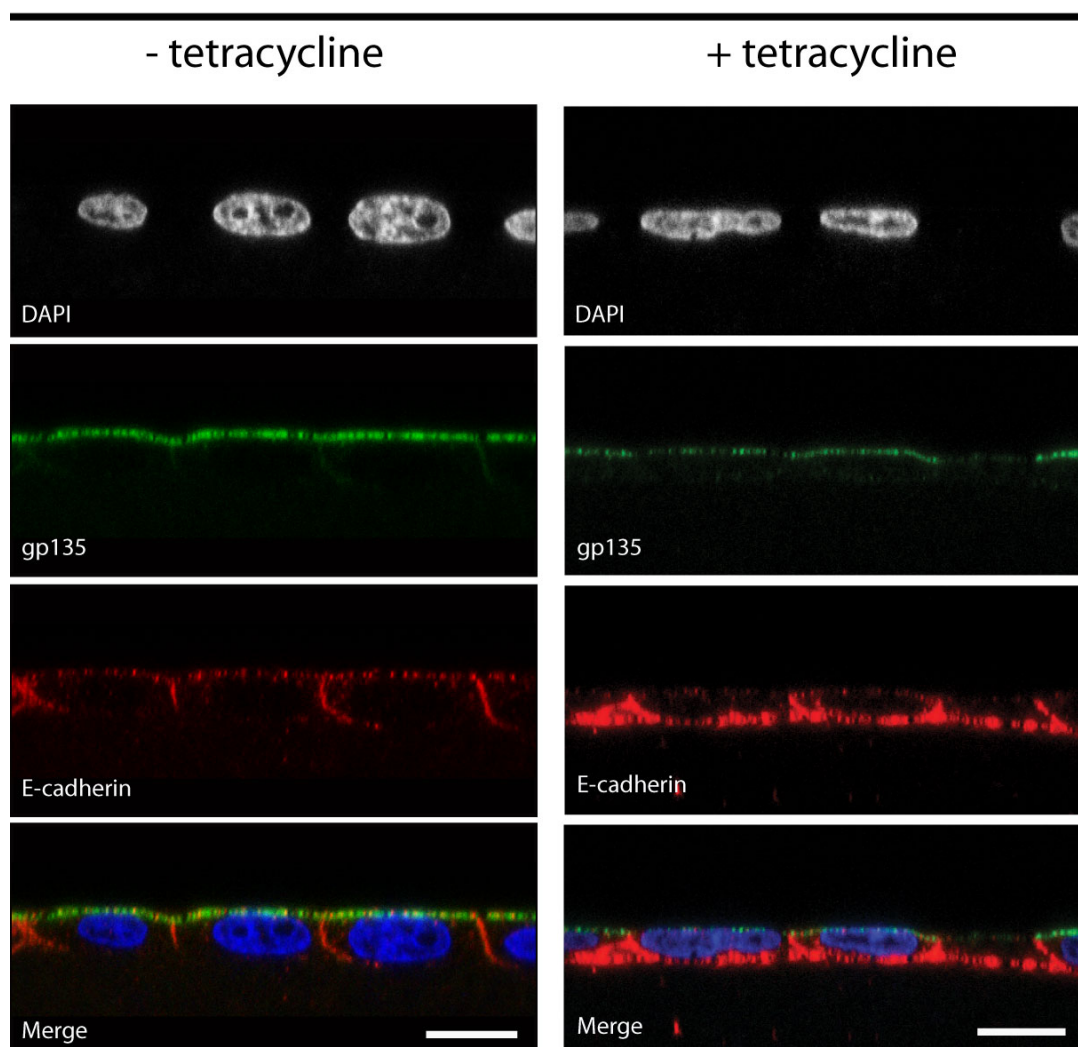


Figure 8 Effect of scribble knockdown on gp135 and E-cadherin localisation. *pTR MDCK scribble shRNA cells were grown on collagen coated coverslips for 64 h in the absence (left hand panels) and presence (right hand panels) of tetracycline. Gp135 is correctly localised in scribble knockdown MDCK cells, but E-cadherin is significantly mislocalised. Scale bars are 10µm.*

3.2.4 Tight junction marker ZO-1 is properly localised in scribble knockdown cells

It has been reported that scribble is a critical mediator of tight junction assembly (Ivanov et al., 2010, Qin et al., 2005b). In MDCK cells, knockdown of scribble slows ZO-1 recruitment to cell-cell contact points and subsequently slows *de*

novo tight junction assembly (Qin et al., 2005b). Any deficiencies in tight junction formation may also affect the interaction of scribble-knockdown MDCK cells with normal MDCK cells. To study tight junctions in pTR MDCK scribble shRNA cells, these cells were seeded onto collagen-coated coverslips and incubated with tetracycline for 64 h prior to fixation and immuno-staining with an anti ZO-1 antibody. XY sections shows that ZO-1 localised to cell-cell contacts correctly and, unlike E-cadherin, the localisation of ZO-1 was tight, as seen in controls without tetracycline (Figure 9, A). XZ sections show that ZO-1 correctly localises between cells to the apical most part of the membrane, demonstrating that after 64 h incubation with tetracycline there is no detectable defect in ZO-1 localisation in scribble-knockdown MDCK cells (Figure 9 B).

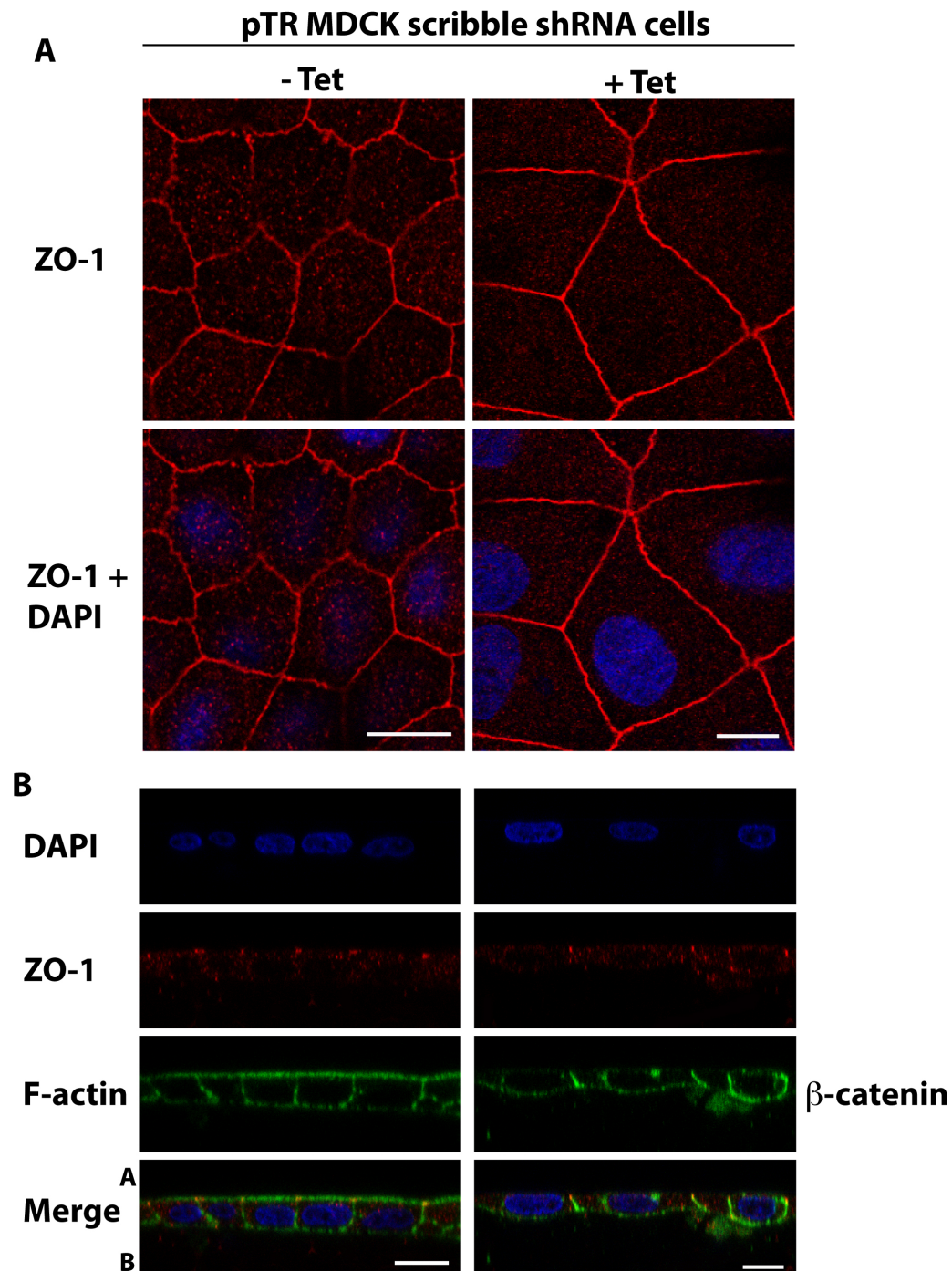


Figure 9 Scribble knockdown does not affect tight junction formation. pTR MDCK scribble shRNA cells were seeded onto collagen coated coverslips and incubated +/- tetracycline (right/left panels respectively) for 64h. Cells were stained with anti-ZO-1 antibody to visualise tight junctions and actin/ β -catenin to visualise cell-cell contacts. Images in A) are of the XY plane, images in B) are of the XZ plane oriented with the apical surface upwards. Note that in correctly polarised epithelial cells, tight junctions are apical to the nucleus so little staining can be

seen in the merged image (A, - tet panels). No defects in ZO-1 localisation can be seen in scribble-knockdown MDCK cells. All scale bars are 10 μ m.

3.2.5 Effect of scribble knockdown on cell polarity in 3D

To further characterise the effect of scribble-knockdown on epithelial cell morphology and to facilitate comparison with previous work (Zhan et al., 2008), the effect of scribble knockdown on 3D cyst formation was analysed. Cells were seeded in type I collagen and allowed to develop into cysts for 13 days before fixation for immuno-fluorescence. pTR MDCK scribble shRNA cells cultured without tetracycline formed cysts with a regular morphology, with gp135 discreetly localised at the apical side of cells (facing the lumen of the cyst) and E-cadherin at cell-cell contacts (Figure 10). When cultured with tetracycline pTR MDCK scribble shRNA cells formed cysts that were larger and less organised than controls without tetracycline, with multi-layering evident (Figure 10). E-cadherin is still found at cell-cell contacts but these cell-cell contacts are not as ordered and linear as in controls incubated without tetracycline. The localisation of gp135 is significantly disrupted in cysts formed from scribble-knockdown cells. Consistent with data from 2D culture, there was no mislocalisation of gp135 to the basolateral membrane (Figure 10). However, the entire lumen stained positive for gp135. This was quite an unexpected finding and has, to my knowledge, not previously been reported. It is unlikely that this stained is from cell debris of apoptotic cells in the lumen, as staining of DNA with Hoechst shows little evidence of apoptotic cells in the lumen.

It is possible that in this assay, scribble-knockdown cells are shedding apical membrane into the lumen, but more experiments are required to make a conclusion about this data. These data are consistent with previously reports that knockdown of scribble has causes mild defects in epithelial morphology in mammalian cell lines (Bilder and Perrimon, 2000, Qin et al., 2005b, Zhan et al., 2008).

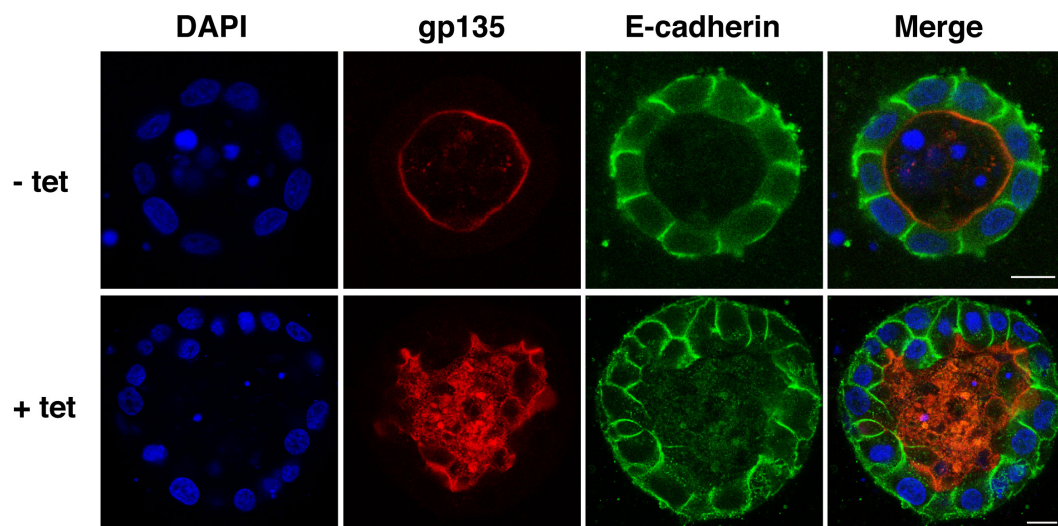


Figure 10 Effect of scribble knockdown on cyst formation. *pTR MDCK scribble shRNA cells were seeded into collagen gels and cultured for 13 days in the absence (top panels) and presence of tetracycline (bottom panels). Cysts formed from scribble-knockdown MDCK cells are larger than controls and have a disrupted morphology. Scale bars are 10 μ m.*

3.2.6 Effect of scribble knockdown on cell proliferation

One of the vital steps in oncogenic transformation is an increase in proliferative capability and a loss of proliferation control. Cell competition was originally identified as the removal of slow proliferating cells from an epithelium (Morata and Ripoll, 1975). Therefore it is important to understand what effect knockdown of scribble will have on the rate of proliferation in MDCK cells.

MDCK control and pTR MDCK scribble shRNA cells were grown on coverslips for approximately 64 hours in the absence or presence of tetracycline. BrdU label was added to cells and incubated for 6 hours before fixation and immunofluorescence. BrdU (bromodeoxyuridine) is a synthetic analogue of thymidine that is incorporated into cells during S-phase and can be detected using immunofluorescence (Gratzner, 1982). The percentage of nuclei that were positive for BrdU staining was analysed and used as a measure of entry into S-phase of the cell cycle. Hoechst was used to stain all nuclei.

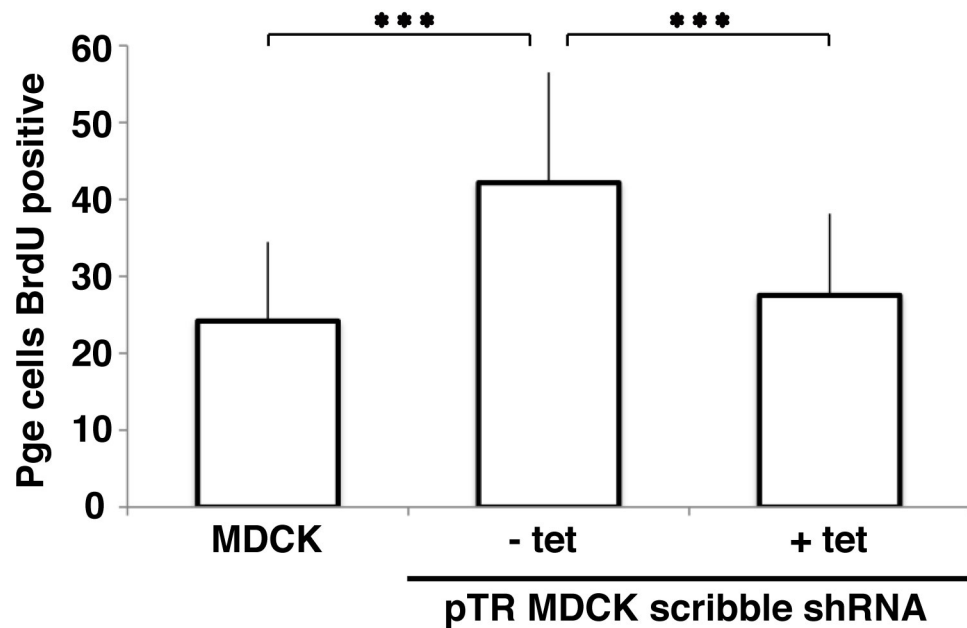


Figure 11 Effect of scribble knockdown on cell proliferation. MDCK and pTR MDCK scribble shRNA cells (+/- tetracycline) were incubated with BrdU for 6 hours before fixing and immuno-staining. The percentage of cells positive for BrdU was analysed. Data are mean of at least three independent experiments. Error bars represent one standard deviation. *** $p < 0.0005$ Student's T-test.

BrdU label was present in 24% of normal MDCK cells and 27% of pTR MDCK scribble shRNA cells incubated with tetracycline (Figure 11). There was no significant difference between these data sets ($p = 0.21$ Student's T-test), showing that there is no significant difference in the rate of entry into S-phase between these two cell types. Unexpectedly, a mean 42% of pTR MDCK scribble shRNA cells incubated without tetracycline were positive for BrdU, a significant increase compared to normal MDCK cells and pTR MDCK scribble shRNA cells with tetracycline.

3.3 Discussion

3.3.1 Scribble knockdown effects polarity of MDCK cells

I have presented data showing that the knockdown of scribble in MDCK cells has mild effects on epithelial cell morphology. From phase contrast images, scribble knockdown cells grown in 2D appear un-polarised but not fibroblastic, consistent with previous reports in MDCK cells (Qin et al., 2005b). Despite these morphological defects and the known importance of scribble in polarity, scribble-knockdown MDCK cells retained correct apical-basal polarity. The apical membrane marker gp135 is properly localised only to the apical membrane and the tight junction marker ZO-1 localises to discreet apical puncta between cells, as expected. The only defect in polarity observed concerns E-cadherin, which is usually found at the basolateral membrane cells; in scribble-knockdown MDCK cells, E-cadherin extends into the basal domain. This has not been observed with other studies in scribble knockdown cells, even studies in MDCK cells that use the same shRNA sequence as used in this study (Qin et al., 2005b). This may be due to the knockdown of scribble being more efficient in the pTR MDCK scribble shRNA cell line than in previously published work, or may have been seen but not reported by other groups (Qin et al., 2005b).

Knockdown of scribble in three-dimensional MDCK cysts showed a slightly more significant defect in epithelial structure (Bilder and Perrimon, 2000). Scribble-knockdown MDCK cells still form cysts with a lumen, unlike cysts made by scribble-knockdown MCF10A cells (Zhan et al., 2008), but signs of multi-layering of cells can be seen and, like in 2D culture, cells lose the regular shape that they have in cysts made from control cells. Cysts made of scribble-knockdown cells are also larger than cysts formed from control cells. This may be due to an increase in cell number in the cyst, or alternatively an increase in cell size. From culture in 2D it can be seen that scribble-knockdown cells are wider and less tall than control cells grown without tetracycline. No direct measurements were made of cell volume, and this is the only true measure of cell size. This would be an interesting further measure of the effect of loss of scribble on epithelial

morphology that may be relevant when scribble-knockdown cells are surrounded by normal MDCK cells.

In summary, knockdown of scribble in MDCK cells produces cells with a less epithelial morphology that nonetheless retain apical-basal polarity. The defects in epithelial morphology are much milder than those seen in *scribble* mutant tissues in *D. melanogaster*, possibly due to the use of shRNA to knockdown scribble protein rather than remove it completely as in *D. melanogaster*. These findings are consistent with previous studies in mammalian cell lines (Dow et al., 2007, Bilder and Perrimon, 2000).

3.3.2 Scribble knockdown effects cell proliferation

Knockdown of scribble in MDCK cells has an uncertain effect on proliferation as measured by rate of BrdU incorporation. pTR MDCK scribble shRNA cells incubated with tetracycline proliferate significantly slower than the same cells incubated without tetracycline. However, scribble knockdown cells proliferate at a rate that is statistically the same as the seen in normal MDCK cells. This is somewhat unexpected, as it has been reported in *D. melanogaster* that loss of scribble can cause aberrant cell proliferation, although loss of tissue size control may contribute to these phenotypes (Johnston and Gallant, 2002, Humbert et al., 2008a, Brumby and Richardson, 2003). Over-expression of scribble in a number of mammalian cell lines represses proliferation as measured by colony formation, BrdU incorporation and entry into S phase (Nagasaka et al., 2006). Knockdown of scribble in the epithelial cell line Caco-2 has been shown to increase entry into S phase (Nagasaka et al., 2006). It is therefore, slightly unexpected that knockdown of scribble in MDCK cells does not increase proliferation as measured by BrdU incorporation, as this is also a measure of entry into S phase. It is not known why pTR MDCK scribble shRNA cells incubated without tetracycline proliferate significantly faster than normal MDCK cells. This has also been seen with other stable cell lines based on pTR MDCK cells (C. Hogan, personal communication). In order to further understand these

data, an alternative clone of the pTR MDCK scribble shRNA cell line could be used. This may clarify the effect scribble knockdown in MDCK has on cell proliferation.

From these data I can conclude that polarity is maintained in scribble-knockdown cells. Also, normal MDCK cells and scribble-knockdown MDCK cells proliferate at a similar level. These data will be important to consider in the next chapter, when the fate of scribble-knockdown cells surrounded by normal MDCK cells is studied.

Chapter 4 Knockdown of scribble causes cell competition in MDCK cells.

Chapter 4 Knockdown of scribble causes cell competition in MDCK cells.

4.1 Introduction

The first results chapter has described the construction of a cell line that stably expresses a tetracycline inducible anti-scribble shRNA, and also the effects of knockdown of scribble on cell morphology, polarity and proliferation of MDCK cells. This chapter will now describe what happens when a small number of scribble-knockdown MDCK cells are surrounded by normal MDCK cells.

Live imaging was used to follow the fate of scribble-knockdown MDCK cells surrounded by normal MDCK cells. This is the best method as it allows the monitoring of the fate of these cells over a long time period (forty hours) rather than at a fixed time point. It also allows observation of live cells in real time from which analyses of cell morphology and cell death can be determined.

This analysis showed that scribble-knockdown MDCK cells die when surrounded by normal MDCK cells, but not when surrounded by other scribble-knockdown cells. Data is presented analysing how scribble-knockdown cells contact normal MDCK cells and signalling pathways activated in scribble-knockdown MDCK cells.

4.2.1 Experimental design

To examine the fate of a small number of scribble-knockdown cells in a normal epithelium, mixed cultures of pTR MDCK scribble shRNA cells and normal MDCK cells were made. pTR MDCK scribble shRNA cells were labelled with red CMTPX dye prior to trypsinisation and mixed 1:10 with normal, unlabelled MDCK cells or non-labelled pTR MDCK scribble shRNA cells (Figure 12). This reliably produces small clusters of stained pTR MDCK scribble shRNA cells surrounded by normal MDCK cells. Cells were allowed to re-establish cell-cell contact and form a monolayer before the addition of tetracycline to knockdown scribble. Controls were incubated without tetracycline. When small molecular inhibitors were used, they were added when medium was changed before live imaging. Live imaging was begun 24 h after the addition of tetracycline and continued for 40 h (making the end point 64 h after addition of tetracycline) with phase images acquired every 10 minutes and fluorescence images acquired every hour. SYTOX® Blue, which passes through damaged cell membranes to bind DNA and fluoresces when excited with blue light, was used to identify dead cells and was added immediately before live imaging. In all time-lapse analyses, a fluorescently labelled scribble-knockdown cell that was removed from the epithelial layer, fragmented into apoptotic bodies and fluoresced blue due to uptake of SYTOX® Blue was counted as dead.

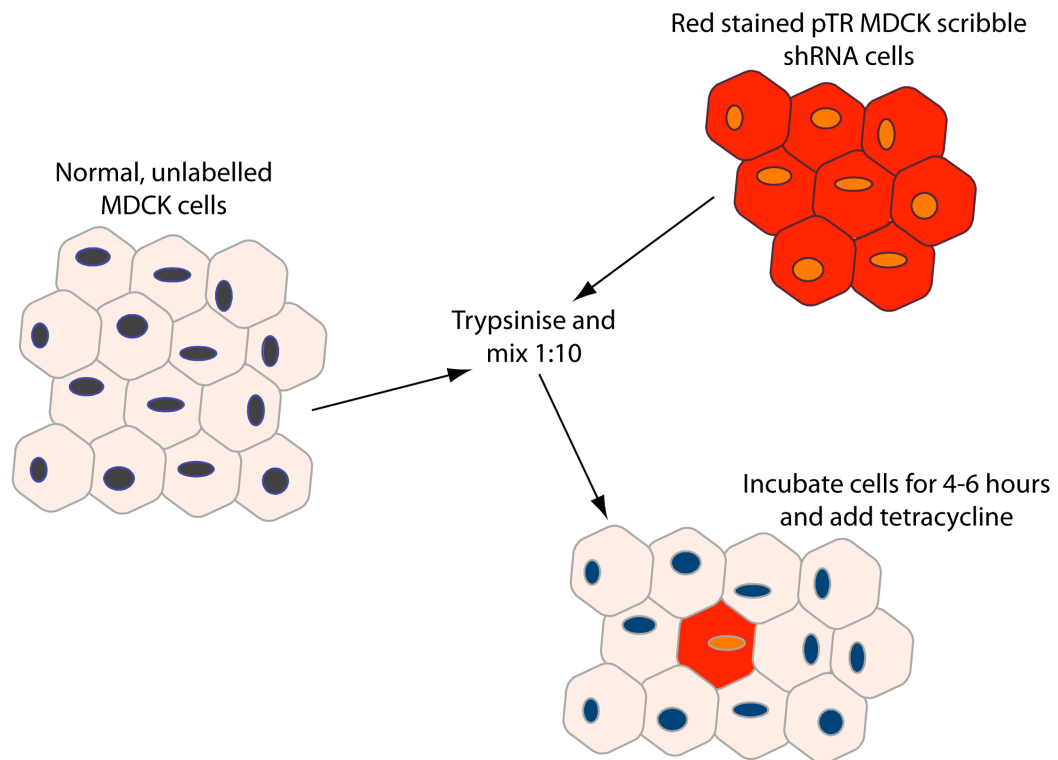


Figure 12 Cartoon of experimental setup. Fluorescently labelled pTR MDCK scribble shRNA cells are mixed 1:10 with normal MDCK cells/unstained pTR MDCK scribble shRNA cells and allowed to form cell-cell contacts and a monolayer before addition of tetracycline to induce knockdown of scribble.

4.2.2 Scribble knockdown cells die when surrounded by normal MDCK cells

At the beginning of time-lapse experiments, 24 h after addition of tetracycline, mixed cultures with and without tetracycline appeared similar, with the labelled pTR MDCK scribble shRNA cells showing no discernible phenotype. Typically, approximately 16 h after the start of time-lapse imaging, some scribble-knockdown cells begin to round up, are apically extruded from the epithelial monolayer and stain positive for Sytox blue; all indications that they have undergone cell death (Figure 13 A, Video 1). At the end of live imaging, after 64 h incubation with tetracycline, approximately 45% of labelled pTR MDCK scribble shRNA cells had died and left the monolayer (Figure 13 A, B and Figure 14). Death of scribble-knockdown cells could be seen when there was a small number of scribble-knockdown cells (Figure 13 A), or a large colony of scribble-knockdown cells (Figure 13 B, Video 2). When large colonies of scribble-

knockdown cells were observed, cell death was predominantly seen at the edge of the colony (Figure 13 B). As these are the cells that have the most contact with normal MDCK cells, it suggests that interaction between the two cell types is important for inducing cell death. In the absence of tetracycline, labelled pTR MDCK scribble shRNA cells (that express normal levels of scribble protein, Figure 15) stayed in the monolayer and did not frequently undergo cell death (Fig 4.2 C and Figure 14). Critically, fluorescently labelled scribble-knockdown cells did not frequently die when surrounded by unlabelled scribble-knockdown cells (Fig 4.2 D and Figure 14), demonstrating that the death of scribble-knockdown cells is dependent on the presence of surrounding normal cells. These data show that in MDCK cells, scribble-knockdown cells are outcompeted by normal cells.

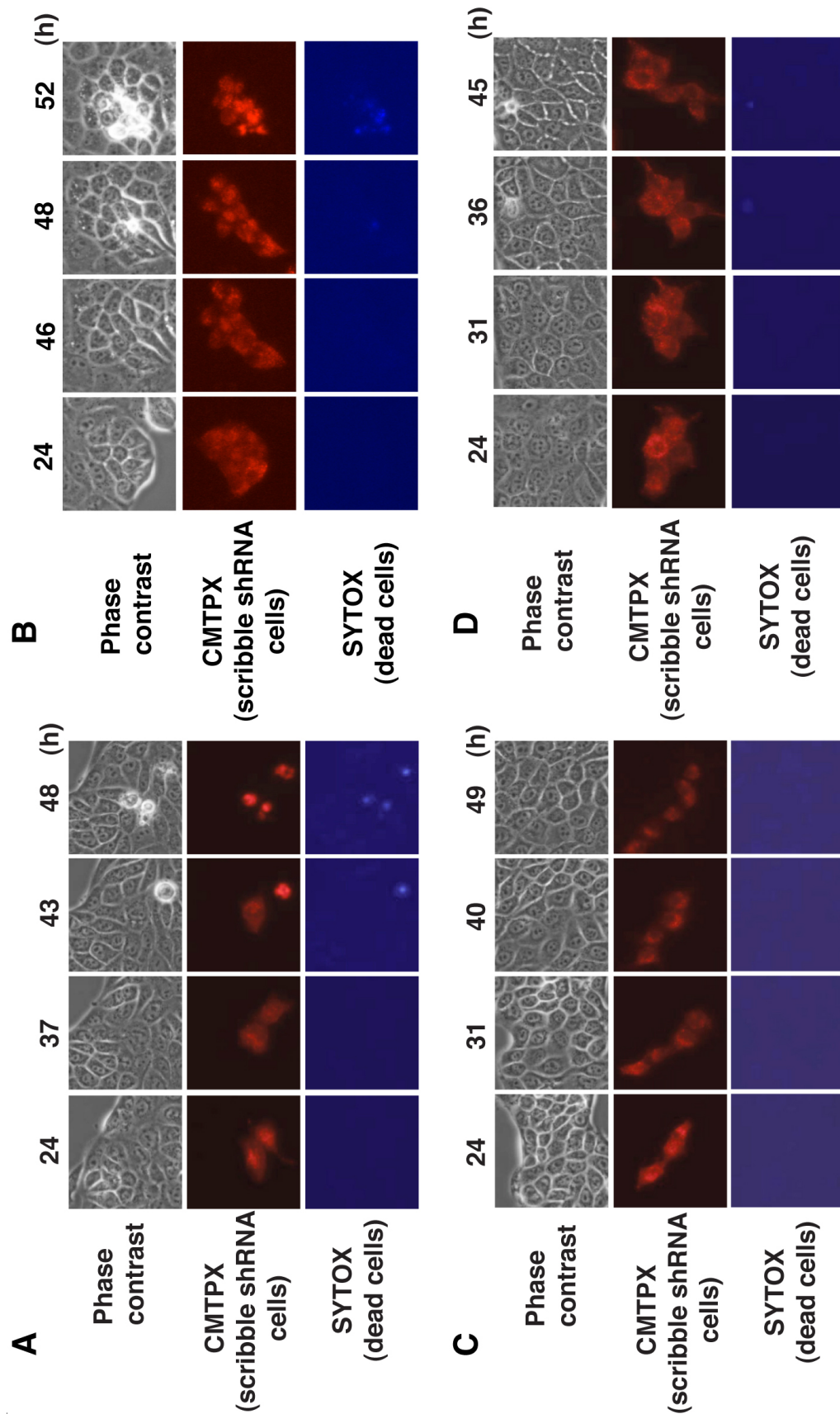


Figure 13 *Scribble* knockdown cells die when surrounded by normal MDCK cells. Red fluorescently labelled pTR MDCK scribble shRNA cells were mixed with

unlabelled normal MDCK cells (A, B and C) and unlabelled pTR MDCK scribble shRNA cells. Tetracycline was added to A, B and D.

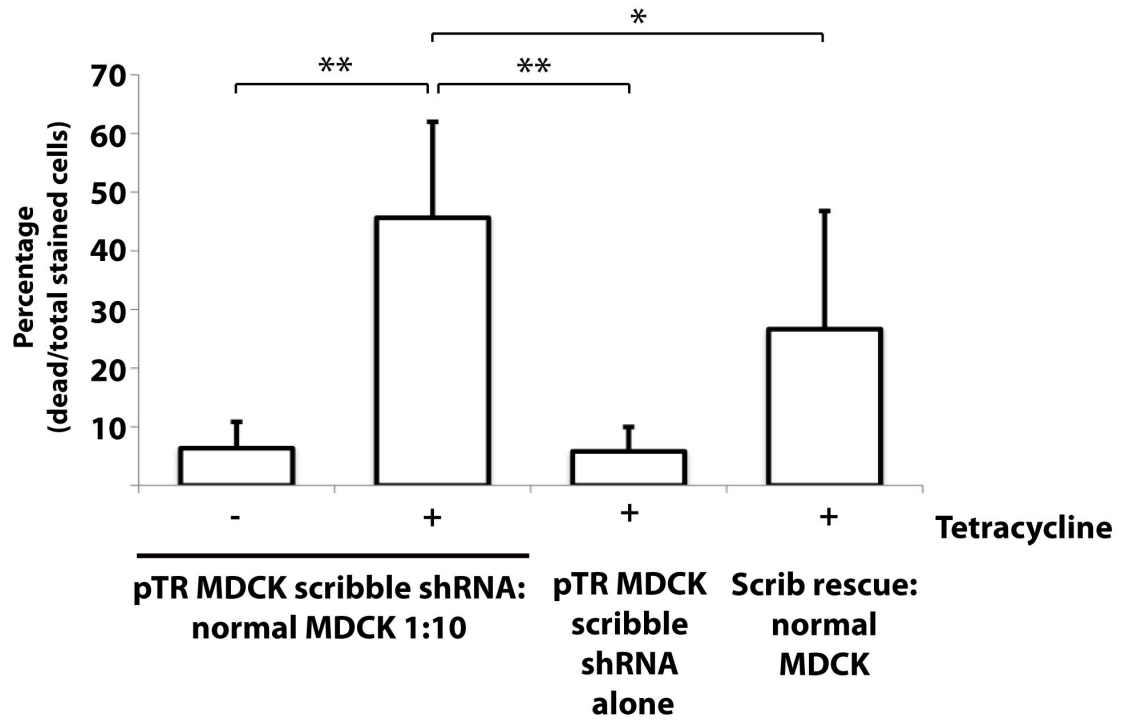


Figure 14 Scribble-knockdown MDCK cells die when surrounded by normal MDCK cells. Quantification of time-lapse data. The number of stained cells that died in the 40h recording was divided by the number of total stained cells at the end of the recording (including dead cells). Data are mean of at least three independent experiments. Error bars represent one standard deviation. ** $p < 0.005$, * $p < 0.05$ Student's T-test.

4.2.3 Expression of hScrib partly rescues cell competition phenotype

In order to demonstrate that death of scribble-knockdown cells when surrounded by normal MDCK cells is in fact due to the loss of scribble protein, a cell line re-expressing scribble was made. This was done so that I could be certain that the phenotype was not due to any off target effects of the anti-scribble shRNA used (Sexana et al., 2003). To restore scribble expression, pTR MDCK scribble shRNA cells were transfected with a construct containing the human version of scribble (pcDNA 4/TO hScrib), which does not contain the exact sequence targeted by the anti-scribble shRNA due to sequence divergence.

Expression of human scribble (hScrib) in this cell line is inducible by tetracycline. Western blotting shows that this cell line, called pTR MDCK scribble shRNA hScrib (scribble-rescue), maintained a level of scribble protein comparable to that of normal MDCK cells after 64 h incubation with tetracycline, a time point at which endogenous scribble is depleted by the expression of the anti-scribble shRNA (Figure 15). The antibody used for this blot detects the human form of scribble, so may have a greater affinity for this than the canine form.

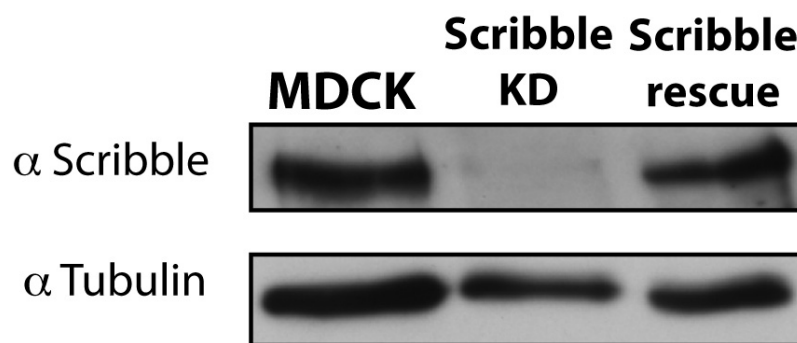


Figure 15 Expression of hScrib in scribble knockdown cells restores scribble protein level. Cell lysates of MDCK, pTR MDCK scribble shRNA (Scribble KD) and pTR MDCK scribble shRNA hScrib (Scribble rescue) cells incubated with tetracycline for 64 h were run on an SDS-PAGE gel. Probing the membrane with an anti-scribble antibody shows levels of scribble protein are similar in scribble-rescue and MDCK cell lysates, and scribble protein is depleted in scribble KD cell lysates. An anti-tubulin antibody was used as a loading control. N.B. The antibody used for this blot detects the human form of scribble, so may have a greater affinity for this than the canine form (in MDCK cells).

Confocal microscopy was used to ensure that over-expressed hScrib was correctly localised. pTR MDCK scribble shRNA hScrib were stained with green fluorescent CMFDA dye, mixed 1:10 with normal MDCK cells and seeded onto collagen-coated coverslips at a density of 2×10^5 per well of a 6 well dish. Cells were incubated with tetracycline for 64 h before fixation and processing for immunofluorescence. Cells were immuno-stained with an anti-scribble antibody. Analysis by confocal microscopy showed that hScrib localises to the cell-cell contact area, although some intra-cellular staining could also be seen that wasn't present in normal MDCK cells (Figure 16). The level of scribble staining in

scribble-rescue cells appears similar to that seen in normal MDCK cells. It has previously been shown that expression of a high level of scribble in MDCK cells inhibits cell proliferation (Nagasaka et al., 2006). This inhibition of proliferation may have an effect on the cell competition phenotype, so this cell line that expresses a level of scribble comparable to normal MDCK cells was selected. Therefore, scribble expression is restored in the scribble-rescue cell line, and scribble is correctly localised. This cell line was then used to see if rescue of scribble protein expression suppresses the cell competition phenotype of scribble knockdown cells (Figure 13).

To observe the fate of scribble-rescue MDCK cells surrounded by normal MDCK cells, time-lapse experiments were set up as described in Figure 12. Expressing shRNA resistant hScrib in scribble-knockdown cells significantly decreased the frequency of cell death seen when they were surrounded by normal MDCK cells (24.6% of labelled cells, Figure 14, $p < 0.05$). This suggests that scribble-knockdown cells die when surrounded by normal MDCK because they lack scribble protein.

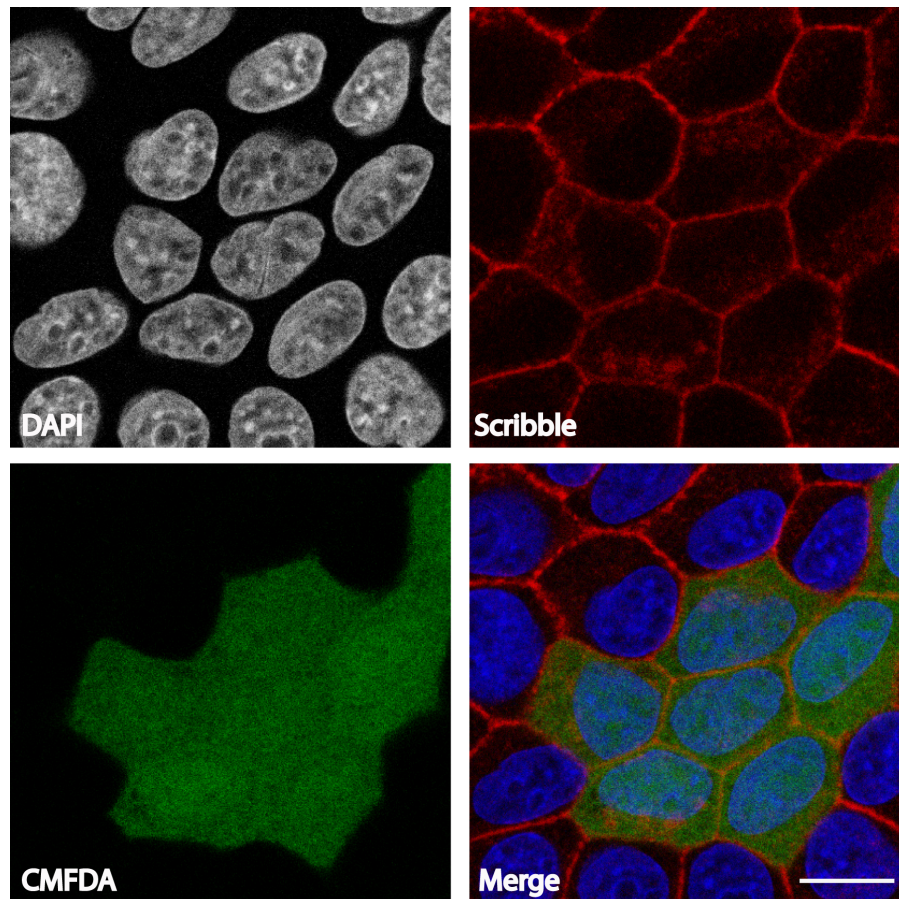


Figure 16 *Scribble is localised to cell-cell contacts in the scribble-rescue cell line. CMFDA stained pTR MDCK scribble shRNA hScrib cells were mixed with normal MDCK cells, plated on collagen-coated coverslips and incubated with tetracycline for 64h. Scribble-rescue cells express scribble at a comparable level to normal MDCK cells and the staining localises to the cell-cell contact area. Scale bar is 10 μ m.*

4.2.4 Experimental design and nomenclature

For subsequent analysis of cells by confocal microscopy, pTR MDCK scribble shRNA cells were labelled with a green fluorescent CMFDA dye and mixed with normal, unlabelled MDCK cells or unlabelled pTR MDCK scribble shRNA cells and seeded onto collagen-coated coverslips. Mixed cultures incubated without tetracycline and pTR MDCK scribble shRNA cells incubated alone with tetracycline were used as controls. Where appropriate, tetracycline was added to

cells for 64 h. All immuno-fluorescence studies were set-up in this way unless otherwise stated.

For the remainder of this thesis, “scribble-KD cells” refers to fluorescently labelled scribble-knockdown MDCK cells that are surrounded by normal, unlabelled MDCK cells. “Scribble-knockdown cells alone” refers to scribble-knockdown MDCK cells surrounded by unlabelled scribble-knockdown cells. The full wording will be used in some cases for emphasis.

4.2.5 Dead scribble-KD cells have activated caspase-3

When surrounded by normal MDCK cells, scribble-knockdown cells are apically extruded and exhibit signs of apoptosis (Figure 13). It is known that apoptotic MDCK cells are removed from the epithelial monolayer very early in the apoptotic process (Rosenblatt et al., 2001). To confirm that apically extruded scribble-KD cells seen in time-lapse images are apoptotic, cells were stained with the apoptosis marker active caspase 3.

Scribble-KD cells frequently contained active caspase-3, although most of the positive staining was in fragmented cells, typical of late stages of apoptosis. Infrequently, intact scribble-KD cells could be seen that were positive for anti-active caspase 3 staining (Figure 17). These were never in the monolayer, always apically extruded as previous work suggests (Rosenblatt et al., 2001). In addition to caspase 3 activation, these cells had condensed chromatin and cytoplasm. This data suggests that scribble-knockdown cells surrounded by normal MDCK cells die by apoptosis.

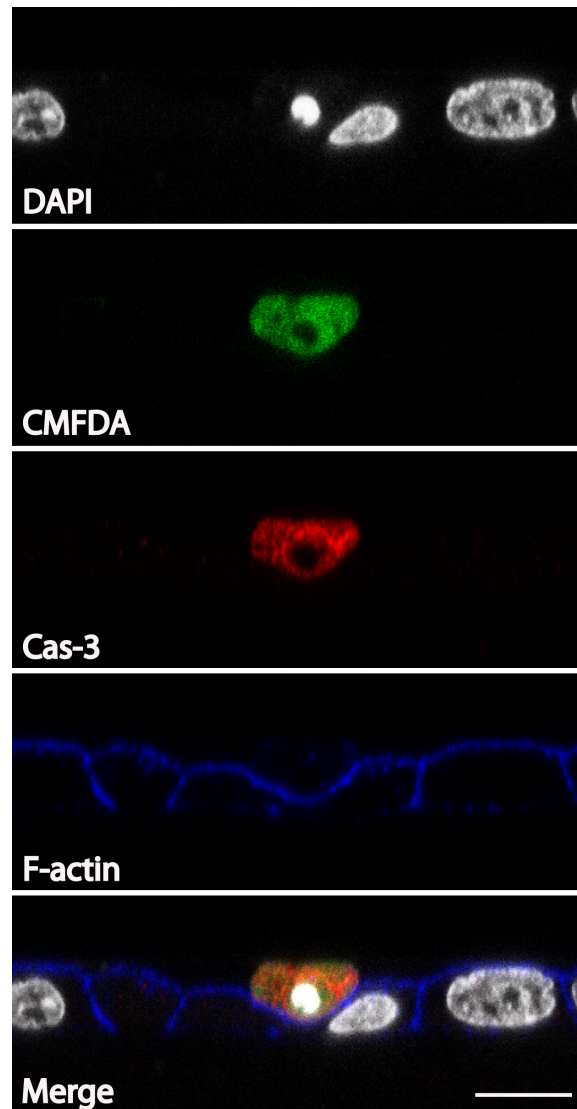


Figure 17 *Dead scribble-KD cells have activated caspase 3.* Green CMFDA labelled pTR MDCK scribble shRNA cells were mixed with normal MDCK cells, incubated with tetracycline for 64 h before fixation with PFA. Cells were immunostained with an antibody that detects activated caspase-3. Scale bar is 10 μ m.

4.2.6 Dead scribble-KD cells have activated Bak and Bax

As well as being essential for energy generation and life of a cell, mitochondria also have an important role in the death of cells (Elmore, 2007). Upon apoptotic signal stimulation, the mitochondrial membrane is permeabilised and pro-apoptosis effectors, in particular cytochrome c, are released from mitochondria (Elmore, 2007). Vital to mitochondrial membrane permeabilisation is the

oligomerisation of Bak (Bcl-2 homologous antagonist/killer) and Bax (Bcl-2 like protein 4), which are activated by numerous stimuli that activate mitochondrial apoptosis (Wei et al., 2001). The exact molecular mechanism of Bak and Bax activation is not understood. When inactive, Bax is located in the cytosol. Upon a cell death-inducing signal, Bax translocates to mitochondria, oligomerizes, becomes an integral membrane protein of the mitochondrial plasma membrane and increases mitochondrial permeability (Desagher et al., 1999, Wei et al., 2001). Bak is slightly different to Bax in that in un-stimulated cells it is already an integral mitochondrial membrane protein, but its activation and the consequence of this is similar to Bax (Cheng et al., 2003, Cuconati et al., 2003). During the activation process and oligomerisation, a specific domain of the N-terminal portion of the Bax is presented, which allows antibody detection of only active Bax (Goping et al., 1998, Desagher et al., 1999, Youle and Strasser, 2008). Bak and Bax are frequently activated together but are not redundant in function (Kepp et al., 2007).

To address whether Bak and Bax are involved in death of scribble-KD cells, cell were immuno-stained with antibodies that identify active forms of these proteins. Scribble-KD cells in the epithelial monolayer show little staining for active Bak or Bax. Extruded cells with fragmented nuclei, a certain indicator of cell death, frequently stained brightly for active Bak and Bax (Figure 18 and Figure 19), suggesting that the mitochondrial apoptotic pathway is activated in dying scribble-KD cells. Dual staining for active Bax and active Bak was not possible as both antibodies were raised in the same species. These data confirm that the intrinsic apoptosis pathway is activated in scribble-KD cells.

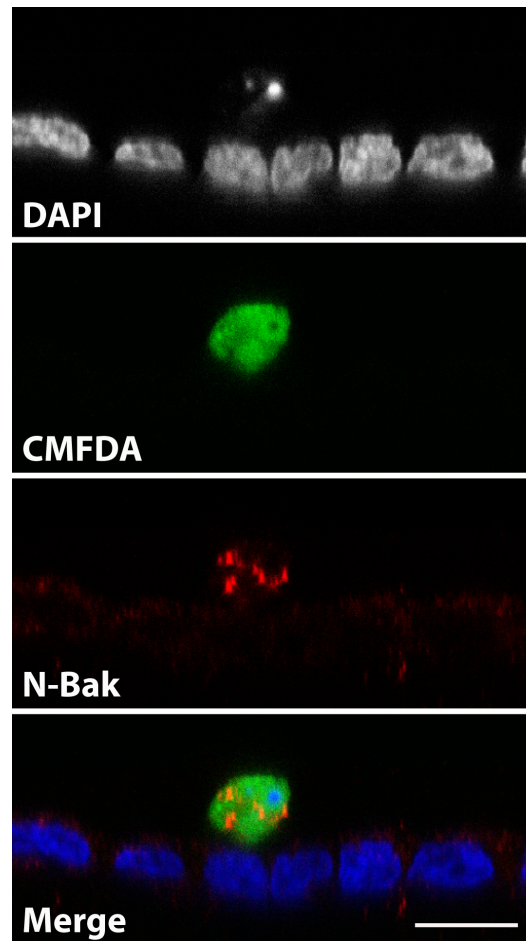


Figure 18 Dead scribble-KD cells have activated Bak. *pTR MDCK scribble shRNA: normal MDCK mixed cultures were incubated with tetracycline for 64 h prior to fixation and immuno-stained with an anti-active Bak antibody. Scribble knockdown cells frequently showed high levels of active Bak staining. Scale bar is 10 μ m.*

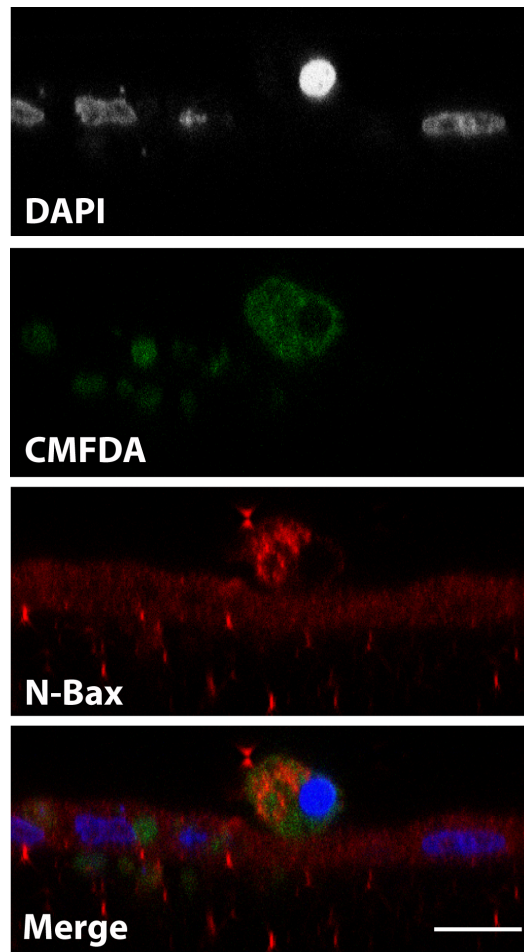


Figure 19 Dead scribble-KD cells have activated Bax. *pTR MDCK scribble shRNA: normal MDCK mixed cultures were incubated with tetracycline for 64 h prior to fixation and immuno-stained with an anti-active Bax antibody. Scribble knockdown cells frequently showed high levels of active Bax staining. Scale bar is 10 μ m.*

4.2.7 Death of scribble-KD cells is not inhibited by Z-VAD-FMK

In *D. melanogaster*, death of *scribble* mutant cells is blocked by inhibition of caspases by over-expression of the baculovirus pan-caspase inhibitor p35 (Brumby and Richardson, 2003). A previous report of apoptosis dependent cell competition in mammalian cells demonstrates that addition of the poly-caspase inhibitor Z-VAD-FMK is sufficient to block cell death (Tamori et al., 2010). Dead Scribble-KD cells have activated caspase 3 (Figure 17), suggesting that caspases are involved in the death of these cells. To see if this was the case, time-lapse

experiments were carried out as described, in the presence of 100 μ M Z-VAD-FMK.

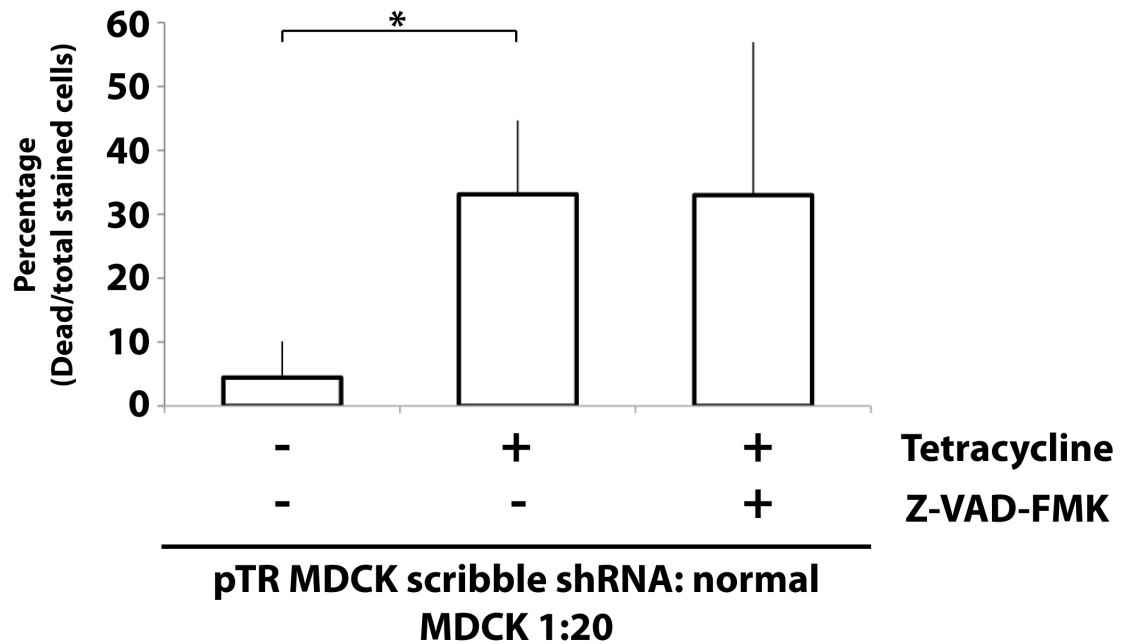


Figure 20 Death of scribble-KD cells is not inhibited by Z-VAD-FMK. CMFDA labelled pTR MDCK scribble shRNA cells were mixed with normal MDCK cells and incubated with tetracycline for 64 h. 100 μ M Z-VAD-FMK was added and the percentage of cells dying under each condition was recorded. Data are mean of three independent experiments. Error bars represent one standard deviation. * $p < 0.05$.

Incubation with Z-VAD-FMK did not reduce cell death of scribble-KD cells. The percentage of scribble-KD cells that died and left the epithelium was virtually identical with and without Z-VAD-FMK (33.1% without Z-VAD-FMK, 33.0% with Z-VAD-FMK). When incubated with Z-VAD-FMK, scribble-KD cells fluoresced blue due to uptake of Sytox dye, indicating they were dead.

Does the failure of Z-VAD-FMK to block death of scribble knockdown cells mean that they are not dying of apoptosis? To try and answer this question, scribble-KD cells were incubated with tetracycline and 100 μ M Z-VAD-FMK and stained with markers of apoptosis.

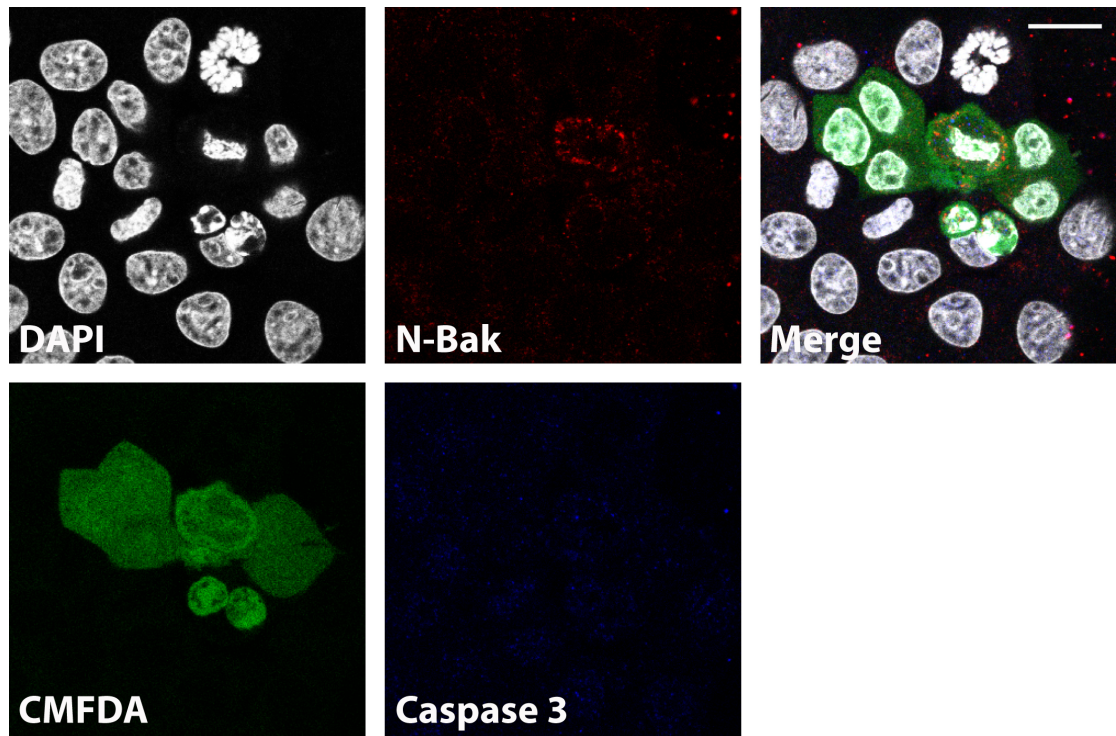


Figure 21 *Scribble-KD cells exhibit apoptotic morphology and activate Bak in the presence of Z-VAD-FMK. CMFDA stained pTR MDCK scribble shRNA MDCK cells were mixed with normal MDCK cells and seeded onto collagen-coated coverslips. Mixed cultures were incubated with tetracycline for 64 h and 100 μ M Z-VAD-FMK for the final 24 h. Cells were stained for active Bak (N-Bak) and active caspase 3. Apoptotic morphology, such as cellular fragmentation, condensation and nuclear condensation was frequently observed. Scale bar is 10 μ m.*

In the presence of Z-VAD-FMK, many dead scribble-KD could still be seen, consistent with live imaging data (Figure 20). Dead scribble-KD cells exhibited apoptotic morphology, with nuclear condensation, cytoplasmic condensation and cell fragmentation frequently observed. Interestingly, activation of Bak and Bax could be seen in dead scribble-KD cells (Figure 21, Figure 22). As expected, no activation of caspase 3 could be seen demonstrating that the expected caspases were inhibited effectively. These data show that in the presence of Z-VAD-FMK, scribble-KD cells are still dying by apoptosis rather than alternative cell death mechanisms, despite the inhibition of many caspases.

Do these data prove that death of scribble-KD cells occurs via a caspase independent pathway? Z-VAD-FMK does not inhibit all caspases completely or equally (Chauvier et al., 2007), which may explain why scribble-KD cells still die in the presence of Z-VAD-FMK. These data will be discussed extensively in the discussion section of this chapter.

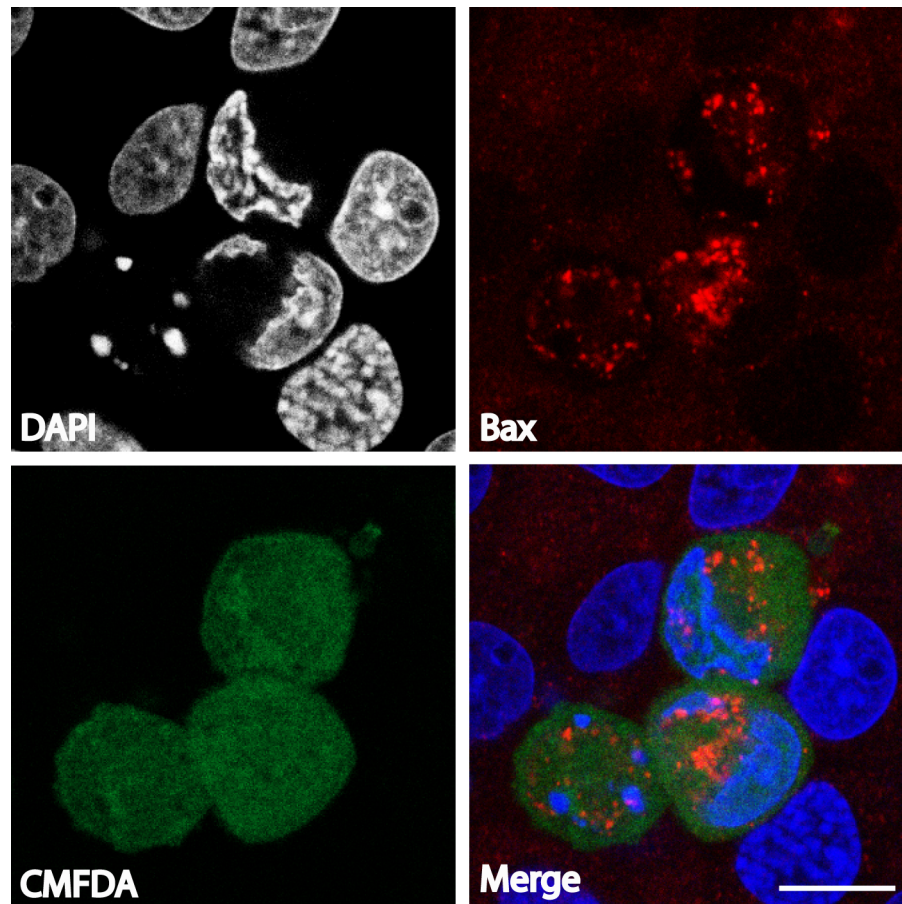


Figure 22 Z-VAD-FMK does not block activation of Bax in scribble-KD cells. CMFDA labelled pTR MDCK scribble shRNA cells were mixed with normal MDCK cells, seeded onto collagen-coated coverslips and incubated with tetracycline for 64 h. 100 μ M Z-VAD-FMK was added for the final 24 h prior to fixation. Active Bax was frequently seen in scribble-knockdown cells. Scale bar is 10 μ m.

4.2.8 Prior to extrusion, scribble-KD cells have altered cell morphology

From analyses of live imaging data, it was observed that scribble-KD cells frequently underwent morphological changes prior to cell death. Prior to cell death, scribble-KD cells appear to pack together more tightly. Also, it was

observed in microscopy analysis that scribble-KD cells were frequently on a slightly different plane to the surrounding normal cells. It has previously been reported that RasV12 or v-Src transformed MDCK cells surrounded by normal MDCK cells have increased apical-basal cell height (Hogan C et al., 2009, Kajita M et al., 2010). To study this, the apical-basal height was measured in scribble-KD cells, the surrounding normal MDCK cells and scribble-knockdown cells cultured alone.

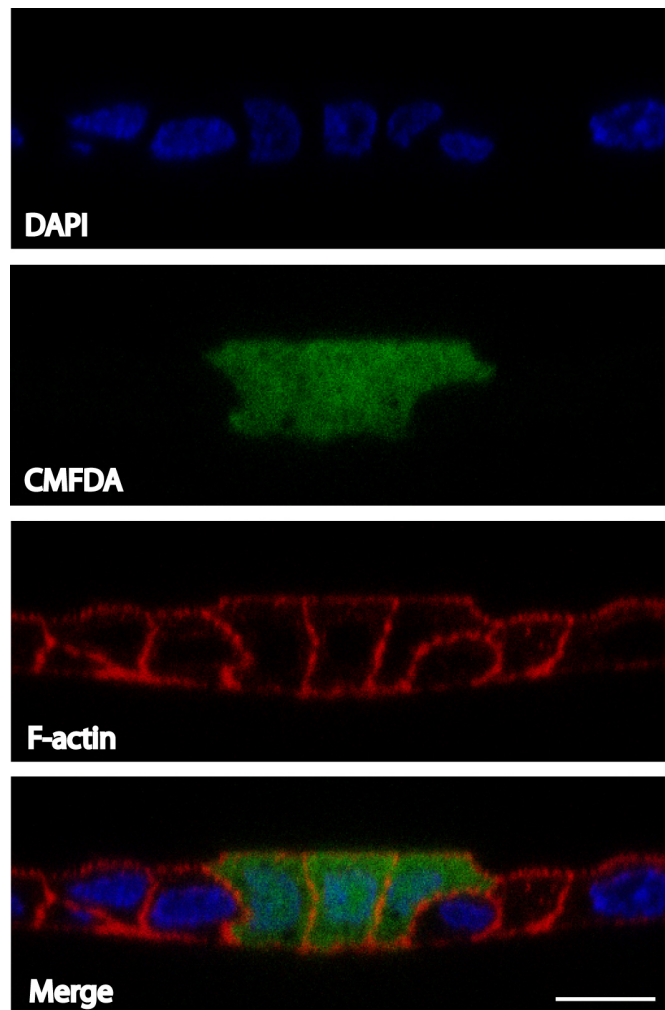


Figure 23 *Scribble-KD cells are taller than neighbouring MDCK cells. CMFDA labelled pTR MDCK scribble shRNA cells were mixed with normal MDCK cells, plated onto collagen-coated coverslips and incubated with tetracycline for 64 h. It was frequently observed that scribble-KD cells were taller than their normal MDCK neighbours. Scale bar is 10 μ m.*

By looking at XZ sections through the epithelial monolayer, it was observed that scribble-KD cells were frequently taller in the apical-basal axis than their normal MDCK neighbours (Figure 23). Quantification of data showed that the mean height of scribble-KD cells was significantly greater than their normal MDCK neighbours (46.1 compared to 31.4 pixels). Conversely, scribble-knockdown cells cultured alone showed the lowest apical-basal height (21.3); there is, therefore, a complete reversion of the reduced cell height phenotype of scribble-knockdown cells cultured alone when they are surrounded by normal MDCK cells (Figure 24).

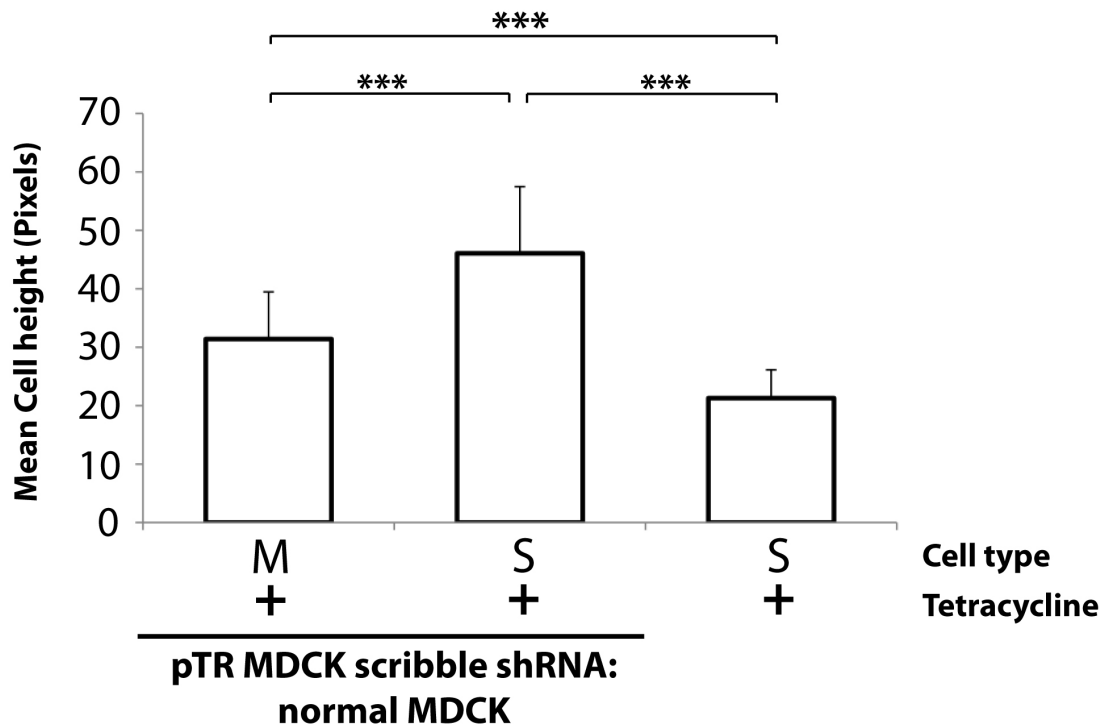


Figure 24 Scribble-KD cells have increased apical-basal cell height. Cell height was measured in XZ images of pTR MDCK scribble shRNA cells and MDCK cells in mixed cultures + tetracycline and scribble-knockdown cells alone. M=MDCK cells, S=pTR MDCK scribble shRNA cells. Data represent mean of three independent experiments, error bars are one standard deviation. *** $P < 0.0001$ Student's T-test.

4.2.9 Cell death of scribble-KD cells is not extrusion dependent

Previous work in the Fujita lab has demonstrated that a single v-Src or RasV12 transformed cell is apically extruded from an epithelial monolayer (Kajita M et al., 2010, Hogan C et al., 2009). From the time-lapse data (Figure 13) and confocal studies (Figure 17) presented here, it is impossible to ascertain if scribble-KD cells die before extrusion or die as a consequence of extrusion. This is an essential distinction to make when considering the molecular mechanism of cell death in scribble-knockdown cells surrounded by normal MDCK cells. Data showing that cell death and extrusion cannot be blocked by the poly-caspase inhibitor Z-VAD-FMK makes this question even harder to answer. Also, scribble KD cells are taller than their normal MDCK neighbours; this is also seen when transformed cells are apically extruded from the epithelium (Hogan C et al., 2009, Kajita M et al., 2010).

To determine if scribble-KD cells can die independently of extrusion, cells were treated with the myosin II inhibitor blebbistatin (Straight et al., 2003), which blocks apical extrusion of apoptotic cells (Rosenblatt et al., 2001). Cells were seeded as described above and blebbistatin was added for the final 24 h of incubation. Cells were fixed and stained for active caspase 3 to identify apoptotic cells. With blebbistatin, apical extrusion of apoptotic cells was blocked and many apoptotic cells (as identified by cytoplasmic condensation, nuclear condensation and caspase 3 activation) could be seen in the epithelial monolayer, something which is very unusual under normal conditions. Under these conditions, 24.6% of stained scribble-KD were positive for active caspase-3 (Figure 25, Figure 26). This is a significant increase when compared to mixed cultures in the absence of tetracycline and scribble-knockdown cells cultured alone (Figure 26). There was a small increase in the percentage of caspase-3 positive cells when scribble-knockdown cells cultured alone, compared to mixed cultures without tetracycline. However, these two data sets were statistically the same ($p=0.056$, two-tailed Student's T-test). These data show that there may be a cell autonomous increase in apoptosis in scribble-knockdown cells that was not identified in time lapse experiments, but it is much smaller than that seen when scribble-KD cells are surrounded by normal MDCK cells. These data also show

that scribble-KD cells die independently of apical extrusion. It is probable that the resulting apical extrusion of scribble-KD cells is because they have entered apoptosis, as previously described in MDCK cells (Rosenblatt et al., 2001).

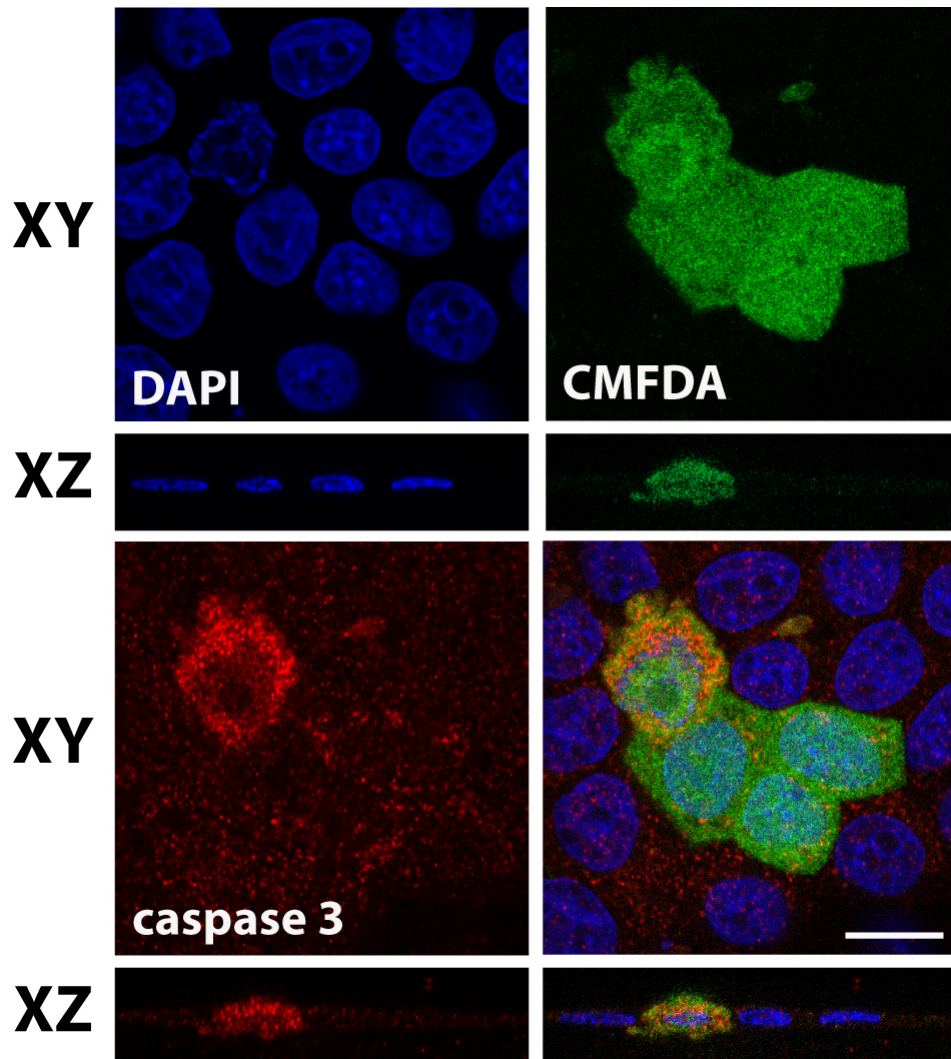


Figure 25 Death of scribble-KD cells is not extrusion dependent. Green CMFDA labelled pTR MDCK scribble shRNA cells were mixed with normal MDCK cells, seeded onto collagen coated coverslips and incubated with tetracycline for 64 h. Blebbistatin was added 24 h prior to fixation. Caspase 3 positive scribble knockdown cells were frequently seen still in the epithelial monolayer. Scale bars are 10 μ m.

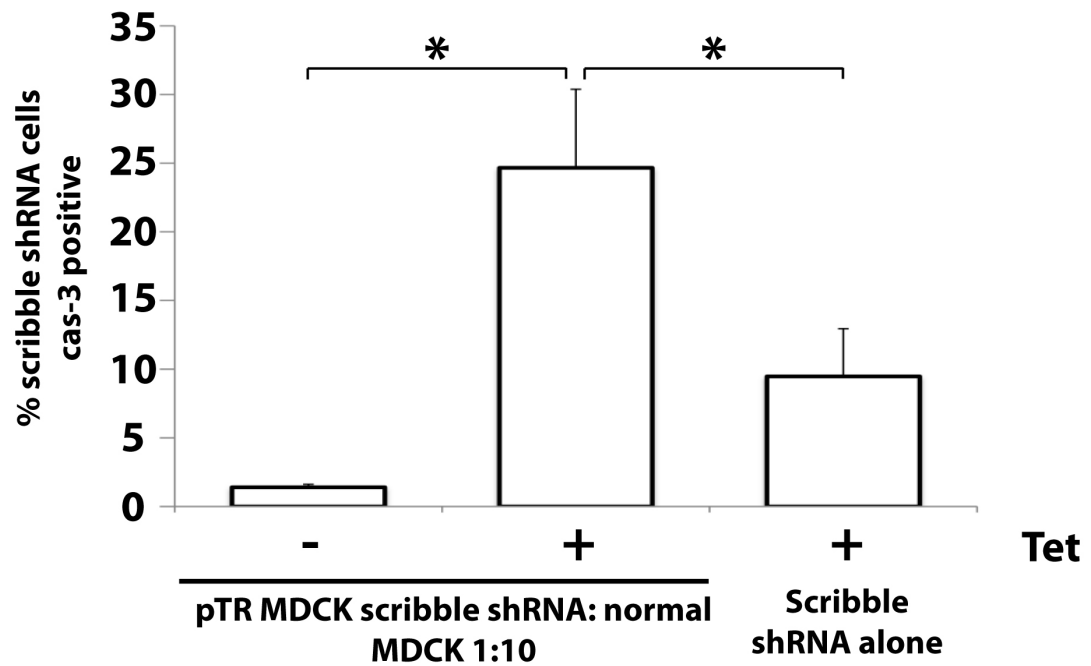


Figure 26 Frequency of caspase 3 positive cells in different cell populations. Stained pTR MDCK scribble shRNA cells were mixed with normal MDCK cells or unlabelled pTR MDCK scribble shRNA cells and incubated with tetracycline (where indicated) for 64 h. Blebbistatin was added to all cells for the final 24 h of incubation prior to fixation. Data are mean of three experiments. Error bars represent one standard deviation. * $p < 0.05$ Student's T-test.

Chapter 4.3 How scribble-KD cells contact their normal neighbours

4.3.1 E-cadherin at cell-cell contacts in scribble-KD cells

The previous chapter (Chapter 3) described a disruption of normal E-cadherin localisation in scribble knockdown cells, something that has also been described previously (Qin et al., 2005). Previous work in the Fujita lab has shown that E-cadherin localisation can be disrupted at the interface of transformed and non-transformed cells (Kajita M et al., 2010, Hogan C et al., 2009). Indeed, differences in E-cadherin localisation and adherens junction function could be an important contributing factor in the death of scribble-KD cells as E-cadherin plays a central role in signalling and morphogenesis of epithelia (Perez-Moreno et al., 2003, Harris and Tepass, 2010). To address a potential role of E-cadherin in the elimination of scribble-KD cells, E-cadherin at the interface between scribble-KD cells and normal MDCK cells was examined.

In mixed cultures without tetracycline, all cells showed an equal level of E-cadherin staining and pTR MDCK scribble shRNA cells were not discernible from normal MDCK cells. Conversely, after incubation with tetracycline, E-cadherin was significantly increased at cell-cell contacts between scribble-KD cells, and also between scribble-KD cells and normal MDCK cells (Figure 27 and Figure 28). E-cadherin in scribble-KD cells localises as a tight, discreet line comparable to E-cadherin in normal MDCK cells (Figure 27). This is in contrast to the disrupted localisation of E-cadherin that was seen in scribble-knockdown cells cultured alone (Qin et al., 2005). Therefore, in mixed cultures with normal MDCK cells there is a rescue of the typical scribble-knockdown E-cadherin phenotype. Also observed was an increase in intra-cellular E-cadherin that localises around the nucleus, which can also be seen in scribble-knockdown cells cultured alone (Figure 27).

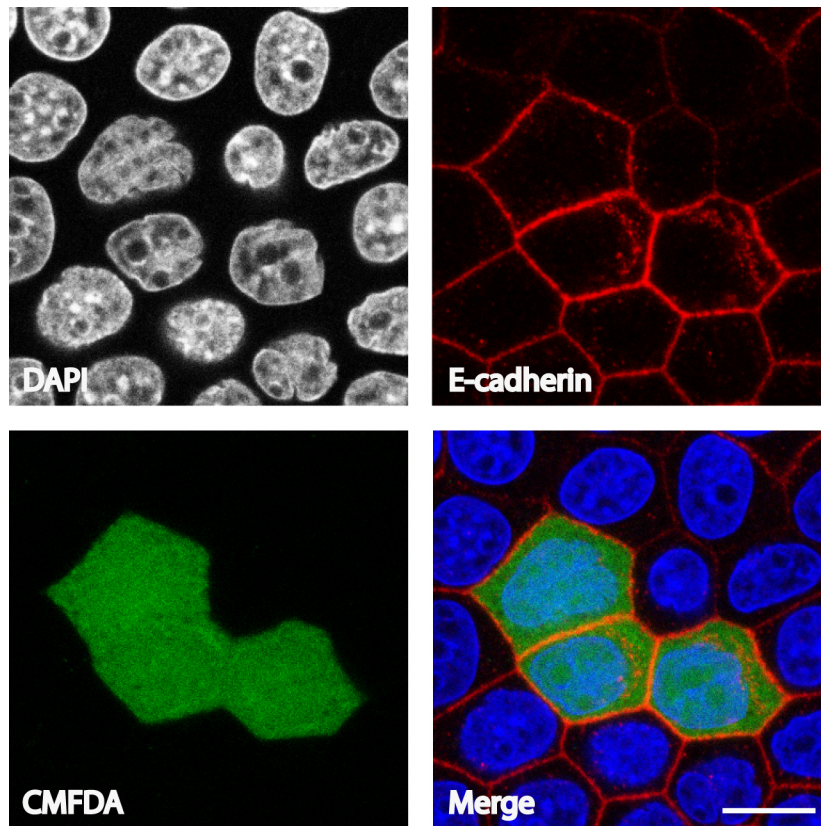


Figure 27 *Scribble-KD cells have higher E-cadherin staining.* Stained pTR MDCK scribble shRNA cells were mixed with normal MDCK 1:10 and incubated with tetracycline for 64h and immuno-stained for E-cadherin. Scribble knockdown cells have very high staining for E-cadherin, especially at cell-cell contacts between adjacent scribble-KD cells. Intra-cellular accumulation of E-cadherin in scribble knockdown cells can also be seen. Scale bar is 10 μ m.

Further analysis of scribble-knockdown cells cultured alone shows that these cells also exhibit high levels of E-cadherin at cell-cell contacts. To examine if there was an increase in junctional E-cadherin specifically in scribble-KD cells, the intensity of E-cadherin staining at cell-cell contacts in XZ images was quantified using Metamorph software (Molecular Devices, CA). This analysis revealed that the intensity of E-cadherin staining at cell-cell contacts between scribble-knockdown cells was the same in scribble-KD cells and scribble-knockdown cells cultured alone (Figure 28). Therefore, the very high E-cadherin staining seen between scribble-KD cells is not induced by the interaction of scribble-knockdown cells with normal MDCK cells. There is a significant

difference in E-cadherin staining intensity at junctions between two scribble-KD cells and between two MDCK cells ($p < 0.005$, Student's T-test). The level of E-cadherin staining at a contact between a MDCK and a scribble-KD cell was also increased compared to MDCK cells, but was lower than that between scribble-KD cells (Figure 27, Figure 28).

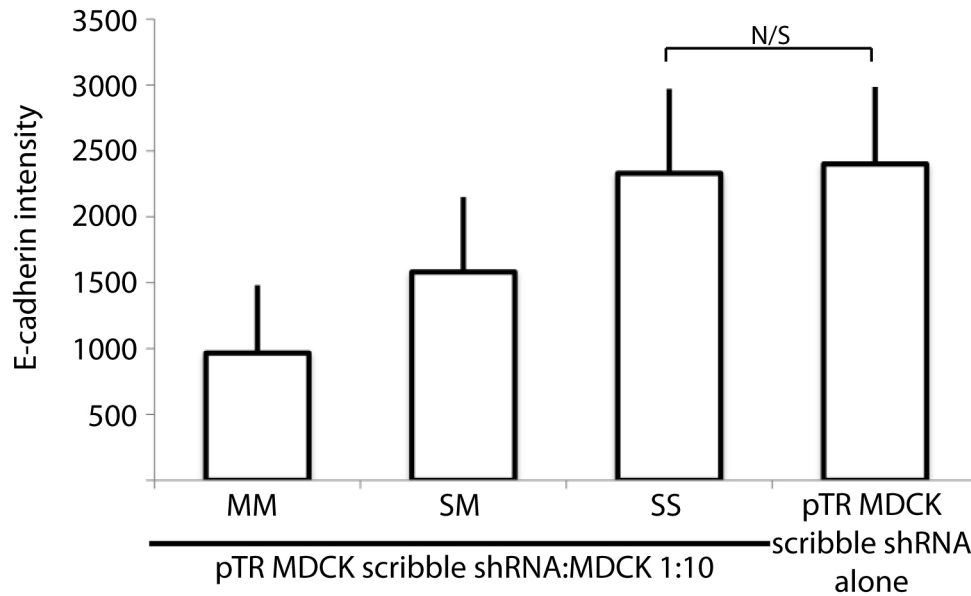


Figure 28 Analysis of E-cadherin staining intensity at cell-cell contacts. Intensity of E-cadherin staining at cell-cell contacts between different cell types in mixed cultures and scribble-knockdown cells alone. MM=Cell-cell contact between two MDCK cells. SM=Cell-cell contact between a scribble knockdown cell and a normal MDCK cell. SS=Cell-cell contact between two scribble knockdown cells. Data are mean of three independent experiments. Error bars represent one standard deviation

4.3.2 Catenins are not enriched in scribble-KD cells

To further examine the nature of cell-cell contacts between scribble-KD cells and surrounding normal MDCK cells, the localisation of β -catenin was studied. β -catenin is a multi-functional protein that can localise to the cytoplasm, the nucleus or cell-cell contacts (Clevers, 2006). E-cadherin directly binds β -catenin

to localise it to the cell-cell contact area and β -catenin is an integral component of adherens junctions (Pokutta S and Wl, 2007). As scribble-knockdown causes an increase in junctional E-cadherin (Figure 28), there should also be an increase in other adherens junction proteins.

As previously observed, E-cadherin staining was increased in scribble-KD cells. However, there was not a corresponding increase in β -catenin staining intensity (Figure 29). Levels of β -catenin appear slightly higher at cell-cell contacts between two scribble-KD cells compared to junctions between two MDCK cells. These data are surprising, as *in vitro* studies have shown that one molecule of E-cadherin binds one or two-molecules of β -catenin (Ozawa and Kemler, 1992) and that the E-cadherin/ β -catenin complex is formed before the proteins reach the plasma membrane (Hinck et al., 1994). Thus, it is unexpected that the enrichment of E-cadherin at cell-cell contacts in scribble-knockdown cells is not accompanied by enrichment of β -catenin. This suggests that E-cadherin at cell-cell contacts in scribble knockdown cells is not properly complexed with catenins.

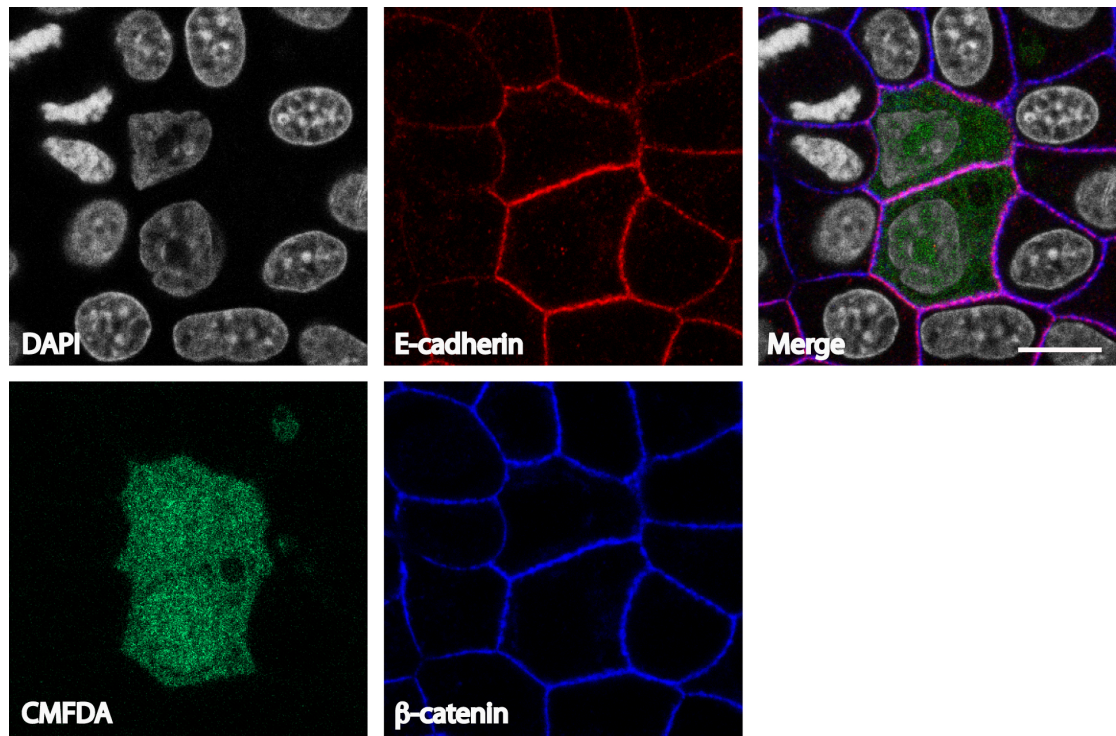


Figure 29 *β-catenin is not enriched at cell-cell contacts between scribble-KD cells. CMFDA labelled pTR MDCK scribble cells were mixed 1:10 with normal MDCK cells and incubated on collagen coated coverslips with tetracycline for 64h. Immuno-staining for E-cadherin and β-catenin shows that the enrichment of E-cadherin does not seem to cause an enrichment of β-catenin at cell-cell contacts between scribble knockdown cells. Scale bar is 10 μm.*

To further examine adherens junctions in scribble-knockdown cells, the localisation of another member of the cadherin-catenin complex, p120 catenin (p120), was studied (Harris and Tepass, 2010). p120 regulates turnover of membrane E-cadherin and is required for maintenance of E-cadherin at cell-cell contacts (Davis et al., 2003). As p120 is a key regulator of E-cadherin at the membrane, it is possible that elevated levels of p120 are responsible for the high level of E-cadherin at cell-cell contacts in scribble-knockdown cells.

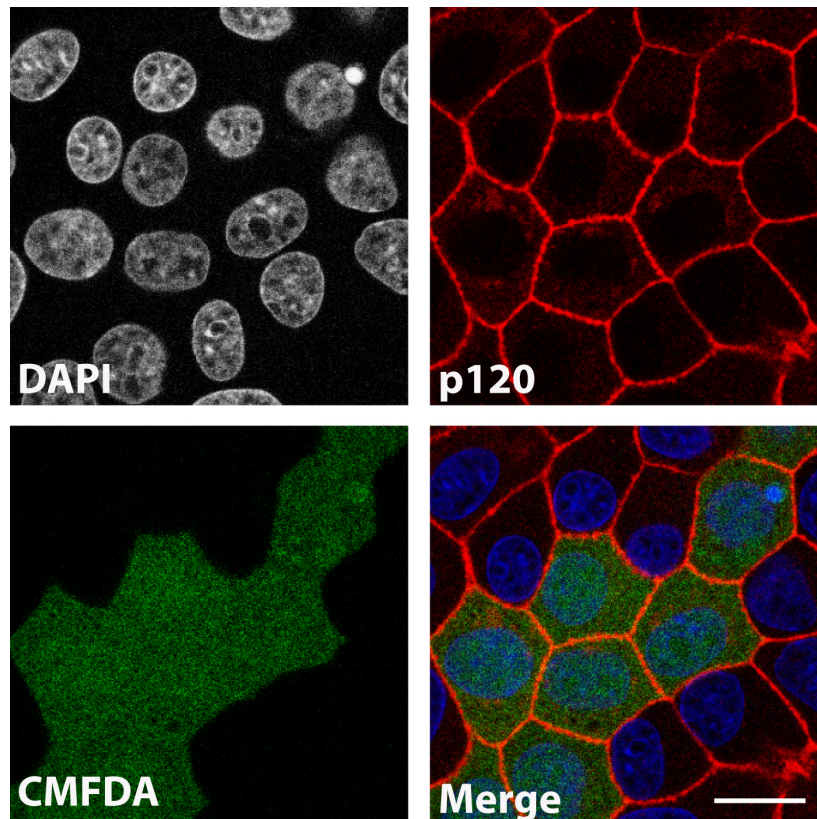


Figure 30 *p120 catenin is not increased at cell-cell contacts in scribble-knockdown cells. CMFDA labelled pTR MDCK scribble cells were mixed 1:10 with normal MDCK cells and incubated on collagen coated coverslips with tetracycline for 64h. Cells were fixed and immuno-stained for p120. There is no increase in p120 at cell-cell contacts in scribble-knockdown cells. Scale bar is 10 μ m.*

Immuno-staining of scribble-KD cells shows that there is no increase in p120 at cell-cell contacts between scribble-KD cells, or between scribble-KD cells and normal MDCK cells (Figure 30). This gives further evidence that despite the increase of E-cadherin at cell-cell contacts in scribble-KD cells, there is not an increase in the cadherin-catenin complex.

If the cadherin-catenin complex is not also increased at cell-cell contacts, then it is likely that signalling downstream of E-cadherin is not significantly increased, as this requires recruitment of catenins (Pokutta S and WI, 2007, Huber et al., 2001). These data will be discussed in the discussion section of this chapter.

Chapter 4.4 Role of JNK and p38 in death of scribble knockdown cells

4.4.1 JNK signalling is not required for death of scribble knockdown cells

It has previously been shown that activation of JNK is required for elimination of *scribble* mutant cells from *D. melanogaster* imaginal wing discs (Brumby and Richardson, 2003, Igaki et al., 2009). In agreement with this, high levels of JNK signalling can be seen in *scribble* mutant cells in *D. melanogaster*, although this may not be dependent on the interaction of *scribble* mutant cells with wild type cells as whole *scribble* mutant tissues also have increased JNK signalling (Pastor-Pareja et al., 2008). In addition, cell death of Mahjong knockdown MDCK cells surrounded by normal MDCK cells is JNK dependent, although activation of JNK in Mahjong knockdown MDCK cells is not reported (Tamori et al., 2010). These data make it important to examine the role of JNK activity in the cell competition induced cell death of scribble-knockdown MDCK cells.

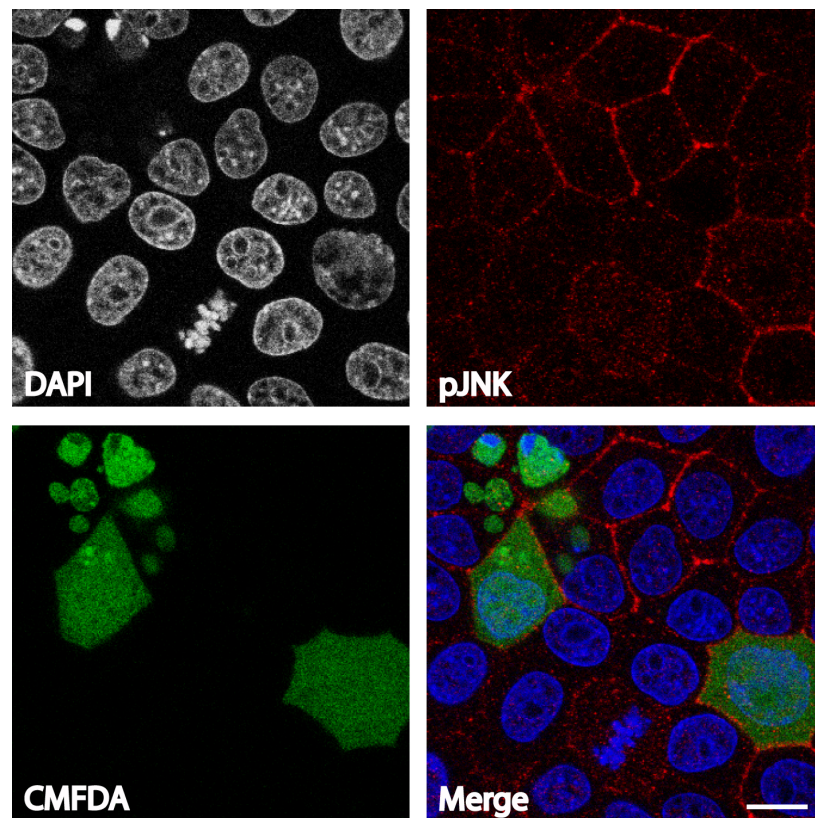


Figure 31 *JNK is not activated in scribble-KD cells.* Mixed cultures were seeded onto collagen-coated coverslips and incubated with tetracycline for 64h. Cells were

stained with an anti phosphorylated JNK antibody (T183/Y185). No increase of staining could be seen in scribble knockdown cells. Scale bar is 10 μ m.

To address the role of JNK in scribble-KD cells, the level of phosphorylated JNK was analysed by immuno-fluorescence with an antibody that only recognises the active, dual phosphorylated (threonine 183 and tyrosine 185) form of JNK.

In all cells, anti-pJNK staining was localised to cell-cell contact areas, especially enriched at contact points between three cells. The staining pattern was variable between experiments, but the image presented is representative of the most common staining pattern (Figure 31). Active JNK was similarly localised in scribble-KD cells and surrounding MDCK cells, indicating that there is no upregulation of JNK activity in scribble-KD cells.

Study of signalling pathways by immunofluorescence allows observation of only a single time point and may not detect dynamic fluctuations of signalling. In order to further study the role of JNK in the cell competition induced death of scribble knockdown cells, SP600125, a specific inhibitor of JNK activation, was used in live imaging experiments (Bennett et al., 2001). Cells were prepared as described in Figure 12. and 5 μ M SP600125 was added prior to live imaging.

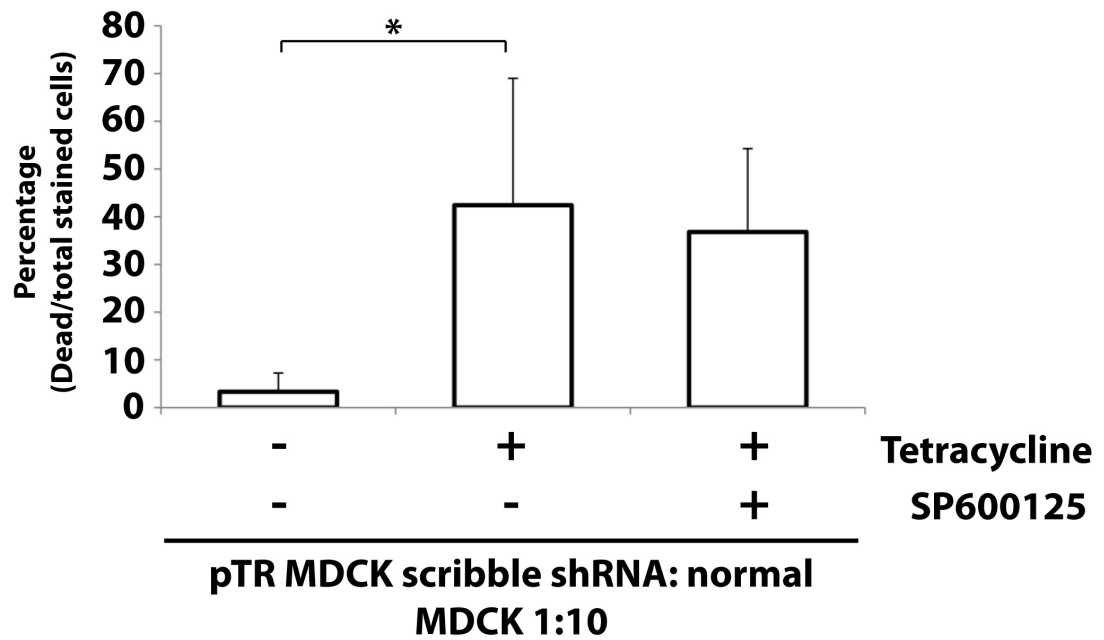


Figure 32 Inhibition of JNK activation does not inhibit death of scribble-knockdown cells. Analysis of live imaging experiments with inhibition of JNK activation. 5 μ M SP600125 was added prior to live imaging for 40h. The percentage of stained scribble-knockdown cells that died during the experiment was the same when incubated with tetracycline and with tetracycline/SP600125. Data represents mean of four experiments, error bars represent one standard deviation. * $p < 0.05$ Student's T-test.

Incubation of cells with a JNK inhibitor did not suppress cell death in scribble-KD cells (Figure 32). When cultured with tetracycline but no inhibitor, 42.4% of stained scribble-KD cells died, consistent with previous experiments (Figure 14 and Figure 20). Addition of the JNK inhibitor SP600125 mildly reduced this to 36.8% of cells, but this is not a statistically significant difference. The inhibitor concentration used in this experiment has been shown to effectively inhibit cell competition induced cell death driven by Mahjong knockdown (Tamori et al., 2010), so it is likely that failure of SP600125 to inhibit death in scribble-knockdown cells is not due to insufficient inhibitor concentration.

4.4.2 p38MAPK is activated in scribble-KD cells

Like JNK, p38MAPK is a MAPK that can be activated to induce apoptosis by a wide variety of cellular stresses (Cai et al., 2006, Bulavin et al., 1999, Ono and Han, 2000, Zarubin and Han, 2005, Kummer et al., 1997). p38MAPK also has roles in other tumour suppressive functions such as contact inhibition and oncogene induced senescence (Faust et al., 2005, Han and Sun, 2007). Unlike JNK, it has not previously been shown to have a role in cell competition induced cell death. As inhibition of JNK activity is not sufficient to inhibit cell death of outcompeted scribble knockdown MDCK cells, the role of p38 MAPK was analysed. In order to examine a role for p38MAPK in the cell death of scribble-knockdown cells, the level of dual phosphorylated p38 MAPK (T180/Y182) was studied using confocal microscopy. When phosphorylated at these residues, p38MAPK is activated and is able to phosphorylate downstream targets such as ATF2 (Raingeaud et al., 1995).

The level of phosphorylated p38MAPK was increased in scribble-KD cells compared to surrounding normal MDCK cells (Figure 33). The level of phosphorylated p38MAPK was also low in scribble-knockdown cells cultured alone, demonstrating that p38 MAPK is specifically activated in scribble knockdown cells only when they are surrounded by normal MDCK cells, a situation which results in death of scribble knockdown cells. To ensure this phenotype is consistent, the staining intensity of phospho-p38MAPK was quantified in scribble-KD cells, the MDCK cells that surround them, and scribble knockdown cells cultured alone. This analysis showed that there was a highly significant ($p < 0.00001$) increase of active p38MAPK in scribble-KD cells, compared to MDCK cells or scribble-knockdown cells cultured alone (Figure 34).

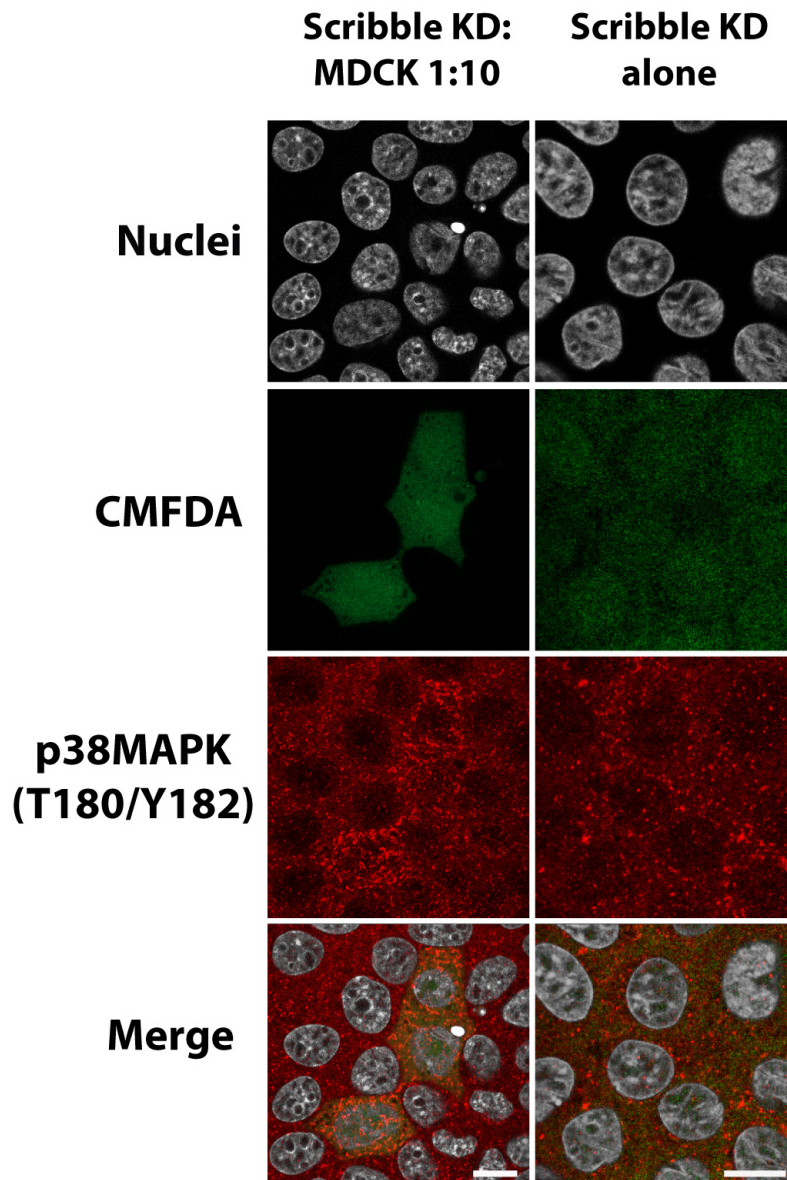


Figure 33 Phosphorylated p38MAPK is increased in scribble-KD cells. Green CMFDA labelled pTR MDCK scribble shRNA MDCK cells were mixed 1:10 with normal MDCK cells or unlabelled pTR MDCK scribble shRNA cells, seeded onto collagen coated coverslips and incubated with tetracycline for 64h. Cells were incubated with an antibody that recognises p38 MAPK phosphorylated at T180/Y182. Scale bars are 10 μ m.

There was no difference in the level of phosphorylated p38MAPK between scribble-knockdown cells cultured alone and MDCK cells ($p=0.36$, Student's T-test). This suggests that the interaction between scribble knockdown cells and

MDCK cells is required for the increase in phospho-p38MAPK in scribble-KD cells.

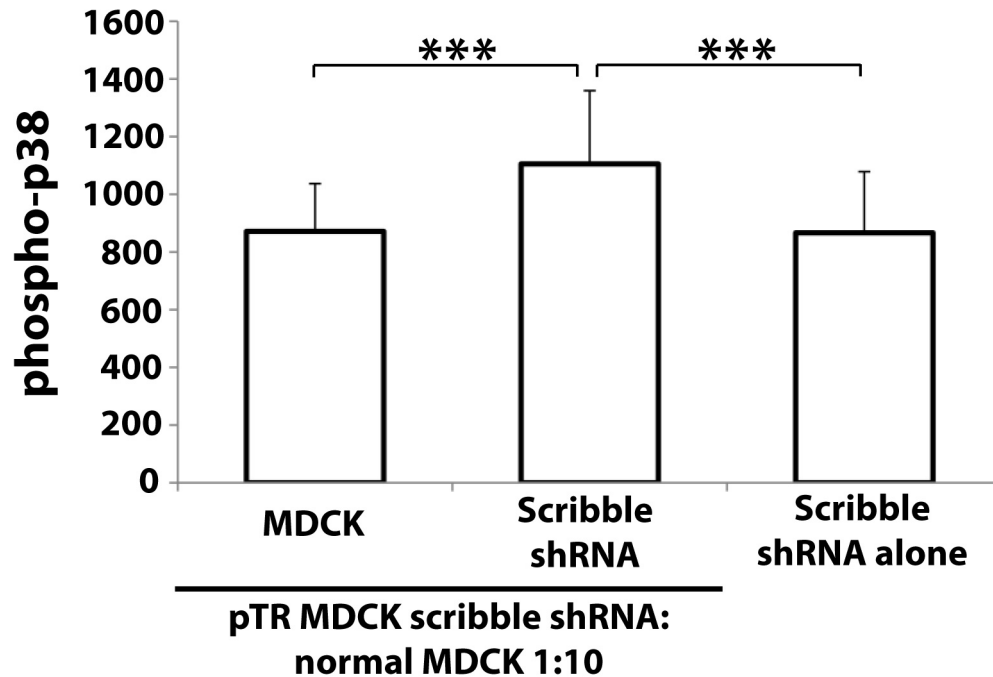


Figure 34 Phospho-p38MAPK is significantly increased in scribble-KD cells. Cells were stained with anti-phospho p38MAPK antibody and immuno-fluorescence intensity was quantified using Metamorph. Data are mean fluorescence intensity of at least 20 cells from each cell type from two independent experiments. Error bars represent one standard deviation. *** $p < 0.0005$.

4.4.3 Inhibition of p38MAPK suppresses cell competition induced death of scribble-KD cells

What is the function of the increased activity of p38MAPK in scribble-KD cells (Figures 33, 34)? As mentioned above, activation of p38 MAPK is known to induce apoptosis downstream of different stimuli. To see if the activation of p38MAPK seen in scribble knockdown cells surrounded by normal MDCK cells is important for the induction of cell death in these cells, the activity of p38MAPK was inhibited using the small molecule inhibitor SB202190 (Manthey et al.,

1998). Time-lapse experiments were set up as described, with 10 μ M SB202190 added to inhibit p38MAPK activity.

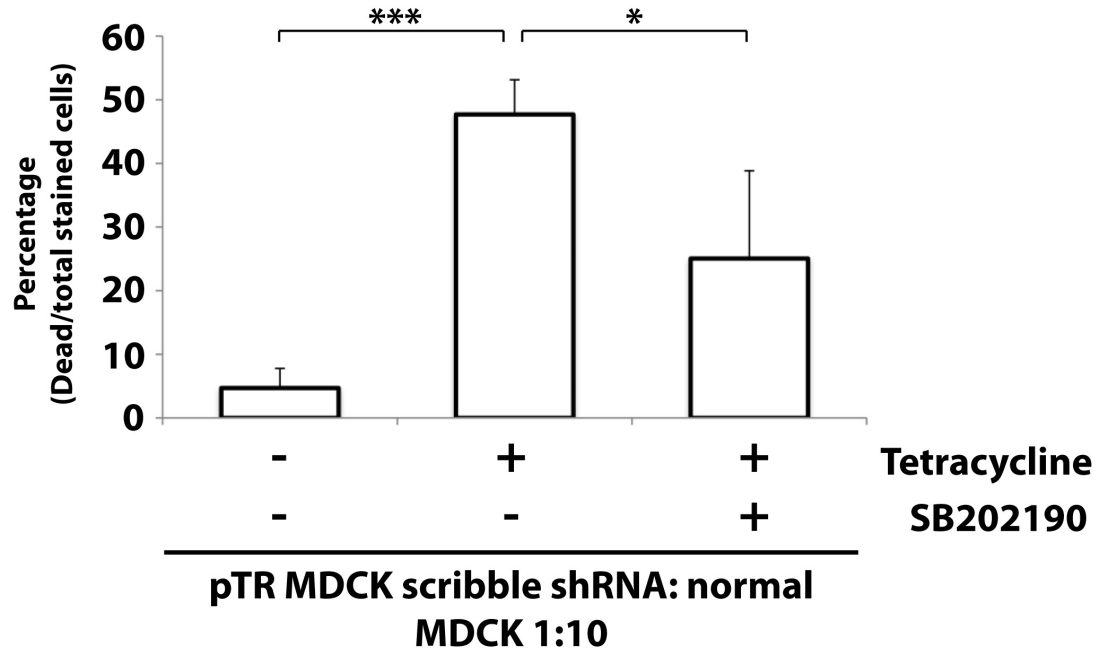


Figure 35 Inhibition of p38 MAPK activity suppresses death of scribble knockdown cells. Labelled pTR MDCK scribble shRNA cells were mixed with normal MDCK cells and incubated with tetracycline and SB202190 as indicated. Data are mean of four independent experiments. Error bars represent one standard deviation. *** $p < 0.0005$, * $p < 0.05$ Student's T-test.

As repeatedly seen in other experiments, very few labelled pTR MDCK scribble shRNA cells in mixed cultures died when incubated without tetracycline (4%). This rose markedly to 49% when tetracycline was added, consistent with previous experiments (Figure 14, Figure 20 and Figure 32). Addition of 10 μ M SB202190 plus tetracycline significantly reduced cell death in scribble-KD cells ($p = 0.0194$, Student's T-test) (Figure 35). This demonstrates that the induction of cell death in scribble-KD requires p38MAPK activity. As an increase in phospho-p38MAPK was seen in scribble-KD cells and very little seen in the surrounding MDCK cells, this suggests that p38MAPK plays a central role in the molecular mechanism of cell death in outcompeted scribble-KD cells.

4.5 Discussion

4.5.1 Scribble knockdown cells die when surrounded by normal MDCK cells

In this chapter, I have shown that scribble-KD cells die when they are surrounded by normal MDCK cells. Scribble knockdown cells incubated alone with tetracycline die at a similar rate to pTR MDCK scribble shRNA cells incubated without tetracycline. Therefore, scribble knockdown MDCK cells show a greatly increased rate of cell death only when surrounded by normal MDCK cells. Re-expression of scribble significantly reduced cell death in scribble-KD cells, suggesting that it is the level of scribble that is responsible for the induction of cell death in scribble-KD cells. Cell death could be seen in scribble-rescue cells when cultured alone; this may explain why there is only a partial suppression of cell death.

With these data in mind the elimination of scribble-knockdown cells from an otherwise normal MDCK epithelial layer can be described as cell competition (Moreno, 2008). This is the first study in mammalian cells of cell competition induced by the knockdown of scribble. Previous work in *D. melanogaster* has demonstrated that clones of *scribble* mutant cells die when in a WT epithelium (Brumby and Richardson, 2003, Igaki et al., 2009). However, an accurate measure of the rate of apoptosis has not been previously made. I have presented data showing that the rate of apoptosis in scribble-knockdown MDCK cells is low, comparable to the rate of cell death in normal MDCK cells. This allows the conclusion that the high rate of cell death seen in scribble-KD cells is indeed due to interaction with normal cells and can be truly termed cell competition.

Cell competition involving apoptosis has only once been described in a mammalian epithelial cell line. Tamori et al have reported the induction of cell competition in MDCK cells by the knockdown of Mahjong, a novel Lgl interacting protein (Tamori et al., 2010). It is interesting that loss of another protein related to the scribble complex also induces cell competition in a mammalian cell line. It is not completely understood how the three proteins of the Scribble complex are

molecularly related with respect to cell competition. In *D. melanogaster*, over-expression of Mahjong is able to suppress cell competition in *lgl*^{-/-}, but not in *scribble* cells (Tamori et al., 2010). This suggests that Mahjong is also not involved in cell competition caused by loss of scribble in MDCK cells.

4.5.2 Dead scribble-KD cells are apically extruded

In epithelial cells *in vitro* and *in vivo*, extrusion and cell death are intricately coupled (Slattum et al., 2009). I have shown that scribble-KD cells have greater apical-basal height than surrounding normal MDCK cells, a phenotype that is also seen in Ras or Src transformed cells surrounded by normal MDCK cells and precedes apical extrusion (Hogan C et al., 2009, Kajita M et al., 2010). However, death of scribble-knockdown MDCK cells surrounded by normal MDCK cells is not dependent on apical extrusion, as inhibition of extrusion in mixed cultures results in a significant amount of caspase 3 positive scribble-knockdown cells present in the epithelium. This means that scribble-KD die before apical extrusion. The increase in cell height of scribble-KD cells may be due these cells already signalling to their neighbours that they are to die and should be apically extruded. A question that was not addressed is whether these scribble-KD cells are larger in total cell volume autonomously, or have specifically increased only their apical-basal height due to interaction with surrounding normal MDCK cells.

These data show that death of scribble-KD cells can occur without extrusion and myosin activity, but they do not rule out a role for such processes in normal conditions. Due to technical limitations, it is very difficult to directly compare the frequency of cell death in scribble-KD cells with and without blebbistatin. Without blebbistatin, the number of dead cells cannot be accurately analysed by confocal microscopy as they are extruded and washed away during the immunofluorescence protocol. The alternative method used in this thesis, live imaging of cells, is also not suitable as in the presence of blebbistatin cells are not extruded and fragmented, both key morphological changes used to quantify cell death in live imaging (along with uptake of Sytox dye). Scribble-KD cells have increased

cell height, and it has previously been shown that this is dependent on myosin activity (Kajita M et al., 2010). The increase in cell height may occur after the induction of apoptosis; previous reports have shown apical extrusion is a very early event in apoptosis of MDCK cells (Rosenblatt et al., 2001). This would agree with the hypothesis that myosin forces are not required for induction of cell death in scribble-KD cells.

4.5.3 Mode of cell death of outcompeted scribble knockdown cells

I have shown that Bak, Bax and caspase 3 are activated in scribble-KD cells. In addition, condensation of chromatin, fragmentation of DNA and cytoplasmic condensation can all be seen in dead cells, strongly suggesting that these cells are dying via apoptosis (Kroemer et al., 2005).

Despite the presence of apoptotic hallmarks and the presence of activated caspase 3, the poly-caspase inhibitor Z-VAD-FMK does not block the death and apical extrusion of scribble-KD cells. This is in contrast to studies in *D. melanogaster* (Brumby and Richardson, 2003) which show that inhibition of caspases blocks cell death induced in scribble mutant cells. In addition, Z-VAD-FMK efficiently blocks apoptosis of Mahjong knockdown MDCK cells surrounded by normal MDCK cells. Despite its reputation as a pan-caspase inhibitor, Z-VAD-FMK does not efficiently inhibit the activity of caspases 2 and 4 (Chauvier et al., 2007, Garcia-Calvo et al., 1998). Caspase 2 is activated early in the apoptotic cascade (Harvey et al., 1997) by multiple different stimuli (Earnshaw et al., 1999). Interestingly, active caspase 2 alone is sufficient to cleave Bid and induce release of apoptosis inducing factors contained in mitochondria, including cytochrome c, apoptosis inducing factor 1 (AIF 1) and Smac/Diablo (Guo et al., 2002). Release of these critical cell death facilitators can result in cell death in the absence of caspase activation, and can also give some of the characteristic morphologies of apoptosis such as DNA fragmentation and chromatin condensation (Susin et al., 1999, Chipuk and Green, 2005).

Activation of Bak and Bax in dead scribble-KD cells shows that the mitochondrial or intrinsic apoptotic pathway is activated. If one of the key caspases that regulates this pathway, caspase 2, is not inhibited by Z-VAD-FMK, then it is understandable that this inhibitor does not prevent death of scribble-KD cells. In support of this theory, active Bak and Bax can be seen in scribble-KD cells in the presence of Z-VAD-FMK.

Both the extrinsic, receptor mediated apoptosis induction pathway and the intrinsic, mitochondrial apoptosis induction pathway can activate Bak and Bax (Fulda and Debatin, 2006). Activation of death receptors by ligands such as TNF or Fas activate caspase 8, which in turn cleaves Bid (Li et al., 1998) which induces oligomerisation of Bak and Bax and mitochondrial outer membrane permeabilisation (Korsmeyer et al., 2000). Detection of active Bak and Bax in scribble-KD cells incubated with Z-VAD-FMK shows that Bak and Bax can be activated independently of caspase 8, as Z-VAD-FMK effectively inhibits caspase 8 activity (Chauvier et al., 2007). This suggests that scribble-KD cells die by apoptosis induced by the intrinsic, mitochondrial pathway. Bax, at least, can be directly phosphorylated and activated by the stress activated kinases JNK and p38 MAPK (Kim et al., 2006). However, from data presented in this thesis it cannot be concluded with complete certainty that induction of apoptosis in scribble-KD cells is via the intrinsic pathway as TNF α may induce Bid cleavage and mitochondrial activation upstream of caspase activation in HeLa cells (Deng et al., 2003). Further experiments are needed to strengthen this conclusion, such as over-expression of the anti-apoptotic protein Bcl-2 in scribble-knockdown MDCK cells. Over-expression of Bcl-2 inhibits the mitochondrial apoptosis pathway, so this experiment would allow a firm conclusion about the role of mitochondrial apoptosis in scribble-knockdown MDCK cells surrounded by normal MDCK cells (Vaux et al., 1988, Youle and Strasser, 2008). Inhibition of apoptosis in scribble-KD cells, but not upstream signalling pathways, would also show what happens when the epithelium cannot induce cell death in scribble-KD cells. In *D. melanogaster* this results in multi-layering and tumour like growth; it would be interesting to see if similar phenotypes are seen in mammalian cell culture (Leong et al., 2009, Brumby and Richardson, 2003).

4.5.4 Scribble-knockdown cells maintain cell-cell contacts with surrounding normal MDCK cells

Previous studies looking at the interaction of a single transformed cell in a normal epithelium in MDCK cells have shown malfunctions of cell-cell contacts between the two cell types. RasV12 transformed MDCK cells surrounded by normal MDCK cells maintain proper E-cadherin based cell-cell contacts with other RasV12 cells, but contacts with normal MDCK cells can be disrupted (Hogan C et al., 2009). V-Src transformed MDCK cells surrounded by normal MDCK cells also have cell-cell contact defects; β -catenin is absent from the apical part of the cell-cell contact between a v-Src transformed cell and a normal MDCK cell (Kajita M et al., 2010). In contrast to these studies, no cell-cell contact deficiencies can be seen in scribble-KD cells. E-cadherin and β -catenin are localised linearly at the cell-cell contact, which is in fact a rescue of the disrupted localisation of E-cadherin in scribble-knockdown cells cultured alone. There are no defects in ZO-1 localisation in scribble-knockdown cells at the time point studied. When surrounded by normal MDCK cells, scribble-KD cells often exhibit a distorted morphology, with a greater apical to basal cell height than neighbouring normal MDCK cells. Even in these instances, which are reminiscent of a phenotype seen when Src transformed cells are surrounded by normal MDCK cells (Kajita M et al., 2010), no cell-cell contact defects could be detected.

In fact, the only cell-cell contact abnormality detected in scribble-KD cells is that they have an increase in E-cadherin located at cell-cell contacts. This increase is not due to the interaction with normal MDCK cells, as the level of E-cadherin at cell-cell contacts is the same when scribble-knockdown cells are cultured alone or with normal MDCK cells. This is unexpected, as scribble has been shown to be required for efficient E-cadherin based cell-cell contact formation (Qin et al., 2005b). There is no similar increase in junctional β -catenin or p120 in scribble-knockdown MDCK cells, whether cultured alone or mixed with normal MDCK cells; in mixed cultures probed with an anti β -catenin antibody, staining intensity

is largely uniform across the two cell types. This suggests that the high level of E-cadherin present at cell-cell contacts in scribble-knockdown cells is not properly functional, as it is not able to properly recruit β -catenin and p120.

Previous, unpublished, work in the Fujita lab has shown that Thrombospondin 1 (Tsp1) binds the extra-cellular domain of E-cadherin (C Hogan, unpublished observations). As will be described in the next chapter, knockdown of scribble protein results in an increase of Tsp1 mRNA and secreted protein. It has previously been described that in polarised MDCK cells, Tsp1 is secreted from the lateral domain (Prabakaran et al., 1999), which is the same sub-cellular localisation as E-cadherin. It is possible, therefore, that Tsp1 produced in scribble-knockdown MDCK cells binds E-cadherin extra-cellularly. As Tsp1 binds the extra-cellular domain of E-cadherin, this could reduce homophilic adhesion between the extra-cellular domains of E-cadherin of neighbouring cells. Without homophilic adhesion of E-cadherin, recruitment of β -catenin and other members of the cadherin complex cannot occur (Niessen and Gottardi, 2008). How Tsp1 binding could immobilise E-cadherin at the cell-cell contact is unknown. It is also essential to note that β -catenin is still recruited to cell-cell contacts in scribble knockdown cells, so some E-cadherin is functioning as expected. The only way to test if Tsp1 is responsible for high levels of E-cadherin at cell-cell contacts between scribble knockdown cells would be to make a double knockdown cell line expressing anti scribble and anti Tsp1 shRNAs. The technical difficulties of this approach are discussed in the next chapter.

In summary, there are no defects in cell-cell contacts between scribble-knockdown MDCK cells and normal MDCK cells. A defect in cell-cell contacts has not previously been reported in *D. melanogaster* models of cell competition caused by knockdown of scribble. This suggests that a deficiency in signalling from adherens junctions is not involved in death of scribble-knockdown MDCK cells surrounded by normal MDCK cells.

4.5.5 The role of JNK in scribble-knockdown induced cell competition

I have presented data that JNK is neither activated in scribble-KD cells, nor required for cell death in these outcompeted cells. This contradicts many published reports of the role of JNK activation in scribble induced cell competition in *D. melanogaster*, as well as the only report of the molecular mechanism of apoptosis dependent cell competition in mammalian cells (Tamori et al., 2010, Igaki et al., 2009, Brumby and Richardson, 2003).

There is evidence that activation of JNK in *scribble* mutant clones in *D. melanogaster* is dependent on the TNF ligand Eiger. The source of Eiger may be epithelial cells or haemocytes recruited to the *scribble* mutant cells (Cordero et al., 2010, Igaki et al., 2009, Igaki et al., 2002). If the source of JNK activating TNF in *scribble* mutant clones is haemocytes, then it is not unexpected that JNK is not activated in a haemocyte free cell culture model of scribble-knockdown induced cell competition.

An alternative source of TNF in *D. melanogaster* experiments is proposed to be the outcompeted *scribble* mutant cells themselves (Igaki et al., 2009). There is little published data to support this mechanism in MDCK cells. It has been reported that MDCK cells contain TNF mRNA, but there is little TNF protein expression when MDCK cells are un-stimulated, or when MDCK cells are stimulated to produce TNF by viral infection (Grone et al., 2002). Of course, these data are in normal MDCK cells and it cannot be ruled out that the knockdown of scribble in MDCK cells facilitates the production of TNF. Published data on the effect of recombinant TNF on MDCK cells also fails to support the hypothesis that outcompeted cells lacking scribble die due to TNF. TNF does not stimulate MDCK cells to produce proteins typical of an inflammatory response, or induce apoptosis in MDCK cells (Feldenberg et al., 1999, Leighton and Pfeilschifter, 1990). It is possible that further unidentified mechanisms also activate JNK in *scribble* cells in *D. melanogaster* that are not present in this cell culture model. Considering these data, along with the failure of JNK inhibition to block cell competition induced death of scribble-knockdown MDCK cells, it seems unlikely

that the TNF-JNK pathway seen in *D. melanogaster* is replicated in this cell culture model of cell competition.

In similar work to that presented in this thesis, the knockdown of the Lgl binding protein Mahjong causes cell competition in MDCK cells. In this system, inhibition of JNK (with the same concentration of inhibitor used in this study) inhibits the death of Mahjong knockdown cells surrounded by normal MDCK cells (Tamori et al., 2010). This suggests that the failure of the JNK inhibitor to inhibit death of scribble-KD cells is not due to a failure of the small molecule inhibitor. These data are from an MDCK model system, so there are the same limitations on TNF induced JNK activation as described; therefore, there is likely to be a TNF independent activator of JNK in cell competition in MDCK cells. It is important to note that Tamori et al do not present data showing activation of JNK in Mahjong knockdown cells, consistent with data shown in this thesis. It is therefore important to perform a positive control experiment to ensure that the anti-phospho JNK antibody is able to detect an increase in phospho-JNK caused by cell stress. This would further support the conclusion that JNK is not activated in scribble-KD cells.

It seems unlikely the molecular mechanism of such closely related models of cell competition could be very different. It is possible that JNK is involved in the elimination of scribble-knockdown MDCK cells, but that inhibition of JNK alone is not sufficient to inhibit death of scribble-knockdown cells surrounded by normal MDCK cells. The data I have presented concerning the role of p38MAPK in the induction of cell death in scribble-knockdown MDCK cells supports the hypothesis that JNK is not central to the mechanism of scribble induced cell competition in MDCK cells.

4.5.6 The role of p38MAPK in scribble-knockdown induced cell competition

I have presented data showing that p38MAPK (p38) is important for the induction of cell death in scribble-KD cells. Activation of p38 is increased in

scribble-KD cells, but is not seen in scribble-knockdown MDCK cells cultured alone. Strengthening the evidence for involvement of p38, inhibition of p38 activity with a small molecule inhibitor significantly suppresses death of scribble-KD cells. Inhibition of p38 does not completely inhibit cell death in scribble-KD cells. An increase in cell death was seen in pTR MDCK scribble shRNA cells surrounded by normal MDCK cells in the absence of tetracycline when cells were incubated with the p38 inhibitor. This suggests that either inhibition of p38 is slightly toxic to cells, or that the inhibitor is not completely specific for p38. Studies in *D. melanogaster* have shown that deletion of p38 makes flies more susceptible to some stresses, so it is possible that this increase in non-specific cell death is due to inhibition of pro-survival functions of p38 (Craig et al., 2004). The role of p38 specifically in scribble-knockdown MDCK cells could be studied by expressing a dominant negative form of p38 in this cell line (Somwar et al., 2002).

I have not been able to address the signalling cascade upstream of p38 activation in outcompeted scribble-knockdown MDCK cells. p38 was initially discovered due to its role in inflammation; consequently its role in immunological responses is best characterised (Lee et al., 1994, Han et al., 1994). In an immunological setting, MKK (mitogen activated kinase kinase) 3, 4 and 6 have been shown to phosphorylate and activate p38MAPK (Ashwell, 2006). There is also a MKK independent pathway that activates p38 (Ge et al., 2002). This makes it difficult to predict what may lie upstream of p38 activation in scribble-KD cells. It is interesting to note that MKK3 and MKK6 only activate p38, whereas MKK4 also activates JNK, which was not seen to be activated in scribble-KD cells (Derijard et al., 1995, Han et al., 1996). The identification of the kinase upstream of p38 in this setting would clarify the role of JNK in the induction of cell death in scribble-KD cells. Inhibition of MKK3, 4 or 6 with small molecular inhibitors, and the study of the levels of phosphorylated MKK3, 4 and 6 with phospho-specific antibodies may help clarify the signalling network upstream of p38. These are experiments I would be keen to perform if time had allowed.

There is considerable overlap in the upstream mechanism that activates p38 and JNK (Tobiume et al., 2001, Wagner and Nebreda, 2009). Given this, it is difficult to understand why increased JNK activity could not be detected in scribble-KD cells. One possible explanation is that p38 activity is suppressing JNK activity. There is some experimental data that supports this hypothesis. JNK activity is increased in mouse embryonic fibroblasts (MEFs) from mice mutant for *Mapk14*, an alternative name for the most commonly studied isoform of p38, p38 α (Hui et al., 2007). It has not been shown that increased activity in p38 inhibits JNK activation. To address the relationship between p38 and JNK, the level of phosphorylated JNK when cells are incubated with the p38 inhibitor SB202190 could be studied.

Activation of p38 is known to induce apoptosis in response to a variety of upstream signals such as inflammatory cytokines, UV irradiation, genotoxic stress and reactive oxygen species (Ono and Han, 2000). p38, and also JNK, is able to phosphorylate the pro-apoptotic protein Bim_{EL} (Cai et al., 2006). This phosphorylation activates Bax and the mitochondrial apoptosis pathway (Putcha et al., 2003). p38 may also be able to phosphorylate and activate Bax more directly (Kim et al., 2006). This suggests that Bax activation in scribble-KD cells may be downstream of p38 activation.

Unlike JNK, p38 has not previously been shown to have a role in cell competition in mammalian cell lines or *D. melanogaster*. There are two isoforms of p38 conserved in *D. melanogaster* and these share many of the same functions as mammalian p38 MAPK, with roles in immunity and response to environmental stress (Craig et al., 2004, Han et al., 1998a, Han et al., 1998b). The role of p38MAPK in apoptosis is not as clear as that of JNK. It has been reported that loss of p38 makes *D. melanogaster* cells more susceptible to certain types of stress (Craig et al., 2004). This does not suggest that deletion of p38 in clones of *scribble* mutant cells in *D. melanogaster* would increase their survival, and may explain why it has not been identified as a suppressor of cell competition in *D. melanogaster*.

4.5.7 Implications of p38 activation in cancer

Like many proteins, p38 has been shown to have pro- or anti-tumourigenic roles depending on the genetic background of the tumour and the stage of tumour progression. p38 is involved in a number of pathways that inhibit tumour progression, including the induction of apoptosis, inhibition of cell cycle progression, contact inhibition and the induction of oncogene induced senescence (Ono and Han, 2000, Ambrosino and Nebreda, 2001, Faust et al., 2005) (Wang et al., 2002). Consequently, p38 has been shown to inhibit tumourigenesis in mouse models of cancer. Mice that lack p38 have less developed lung epithelia and are more susceptible to tumour formation in response to expression of oncogenic Ras (Ventura et al., 2007). Deletion of p38 in the liver also makes mice more susceptible to tumour formation (Hui et al., 2007). These data have led some to term p38 a tumour suppressor (Bulavin and Fornace, 2004). Genomic studies of human tumours have identified mutations in kinases upstream of p38 activation, particularly MKK4, suggesting that p38 activity may be reduced in some human cancers (Parsons et al., 2005, Su et al., 1998).

In some circumstances, p38 has also been shown to be pro-tumourigenic. Experiments in mammalian cell culture have shown that p38 is required for the invasion of tumour cells from different origins (Hsieh et al., 2007, Junttila et al., 2007). Consistent with a pro-tumourigenic role in the right setting, p38 is expressed at high levels in some human tumours (Pomerance et al., 2006, Greenberg et al., 2002, Junttila et al., 2007).

Considering the varied role of p38 in cancer, the implications of upregulation of p38 activity in scribble-KD cells on tumourigenesis are difficult to determine. The aim of this thesis is to model the early stages of tumourigenesis. Data presented in this thesis shows that activation of p38 contributes to the elimination of scribble-knockdown MDCK cells from a normal epithelium. In this scenario p38 is acting as a tumour suppressor. It is possible that in later stages of

tumour progression, where cells that lack scribble have been able to prevent cell death, p38 activity may be able to promote metastasis. Important to consider is that in these later stages of tumour development, cell-cell contact between transformed cells and non-transformed cells may be minimised and p38 may not be activated in cells with low scribble protein expression. Further study of the function of scribble in mouse models of cancer will help to understand any link between p38 activation and loss of scribble protein.

4.5.8 Conclusion

Scribble-knockdown MDCK cells in an epithelial monolayer of normal MDCK cells die due to cell competition. Activation of Bak and Bax independently of caspase-8 in dead scribble-KD cells suggests that these cells are dying due to the intrinsic apoptosis pathway. No signs of cell-cell contact deficiencies would be seen between scribble-knockdown cells and normal MDCK cells. The observed cell death of scribble-KD cells can occur independently of apical extrusion and is p38MAPK, but not JNK, dependent.

Chapter 5. Knockdown of scribble activates Tsp1 transcription and TGF- β signalling

Chapter 5. Knockdown of scribble increases Tsp1 transcription and TGF- β signalling

5.1.1 Introduction

This chapter describes the identification of thrombospondin 1 (Tsp1) as a protein that is up-regulated in scribble-knockdown MDCK cells. The upregulation of Tsp1 in scribble-knockdown MDCK cells was identified by the screening of conditioned culture medium. The identification of Tsp1 was confirmed by Western blotting and qPCR analysis of mRNA levels shows that Tsp1 is upregulated at the mRNA level in scribble-knockdown MDCK cells.

One of the key biological functions of Tsp1 is the activation of latent TGF- β . It is shown that scribble-knockdown MDCK cells have increased phosphorylated SMAD2, an indication that the TGF- β signalling pathway is activated. The implications for these data in the cell death of scribble-knockdown cells when surrounded by normal MDCK cells is then examined.

I will first introduce some of the biological functions of Tsp1 that are relevant for work described in this chapter.

5.1.2 Structure and function of Thrombospondin 1

Thrombospondin 1 (Tsp1) was first identified from human platelets (Lawler et al., 1978). It is a large, multi-domain glycoprotein that forms a native extracellular homotrimer (Adams, 2001). The thrombospondin family is a highly evolutionarily conserved protein family that consists of five proteins, of which thrombospondin 1 and 2 share significant sequence homology and form the A subgroup (Bentley and Adams, 2010). Thrombospondin 3, 4 and 5 form the B subgroup (Adams, 2001). Family members have distinct but not exclusive expression profiles (Adams, 2001). A crucial point for this study is that Tsp1 is expressed endogenously and secreted in MDCK cells (Adams, 2001, Prabakaran et al., 1999).

Tsp1 is a large, 1170 amino acid protein with many protein domains, and has been shown to have roles in many different biological processes (Bornstein, 1995) (Figure 36). The type 1 repeats, of which there are 3, are responsible for one of the key functions of the Tsp1, the activation of latent TGF- β , and are exclusive to subgroup A thrombospondins (Lawler, 2000). The type 2 and type 3 repeats and the carboxy terminal are found in all thrombospondins (Adams, 2001).

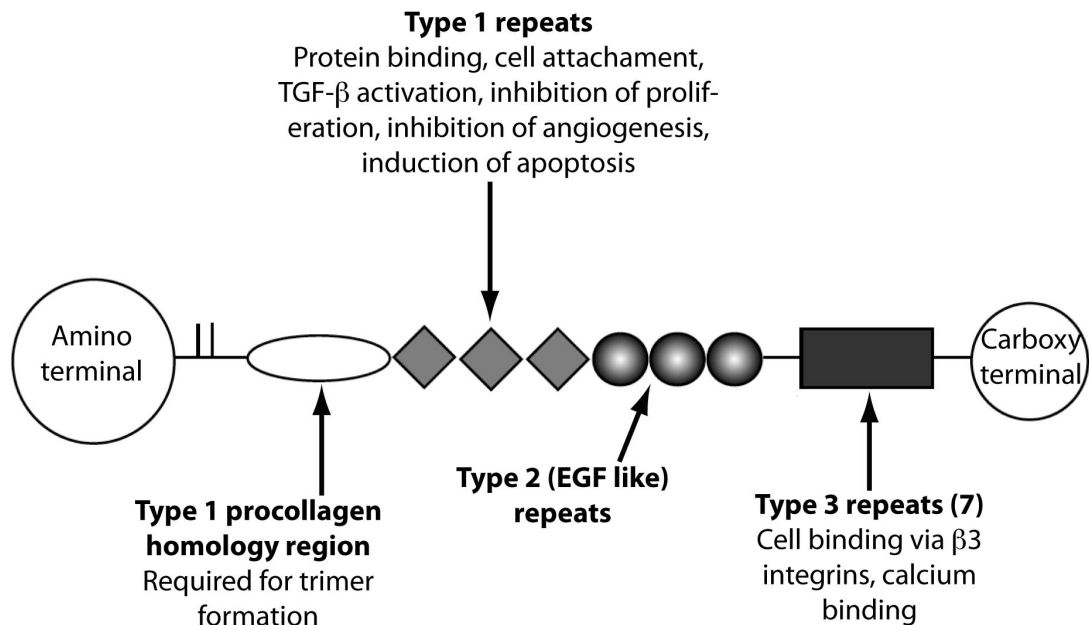


Figure 36 Domain map of thrombospondin 1. The domains of thrombospondin 1 are highlighted, along with some of the binding partners of each domain. Adapted from (Bornstein, 1995) and (Lawler, 2000).

One of the principal characteristics of Tsp1, and one that has long been of interest to biologists and pharmaceutical companies, is its ability to extracellularly activate latent TGF- β (Murphy-Ullrich et al., 1992). TGF- β is synthesised as a part of a latent precursor molecular that requires activation before it can bind receptors and exert its biological functions (Lawrence et al., 1984). A short, four amino acid residue (LSKL) in the latency-associated peptide (LAP) of inactive TGF- β associates with a RFK sequence in the first type 1 repeat in Tsp1. This changes the conformation of inactive TGF- β , allowing it to bind to receptors and exert its biological functions (Ribeiro et al., 1999). Tsp1 is required for activation of TGF- β in certain developmental programmes in vivo,

notably lung and pancreas development (Crawford et al., 1998), but there are other activators or latent TGF- β and Tsp1 is not always required for TGF- β activation (Abdelouahed et al., 2000).

Another critical and medically important function of Tsp1 is the inhibition of angiogenesis. Tsp1 was in fact the first naturally occurring angiogenesis inhibiting peptide discovered (Good et al., 1990), a function it shares with thrombospondin 2 (Kyriakides et al., 1999). The anti-angiogenic ability of Tsp1 is due to selective induction of apoptosis in endothelial cells. Studies of corneal neovascularization have clarified the molecular mechanism by which Tsp1 blocks growth of blood vessels (Jiménez et al., 2000). Inhibition of neovascularization by Tsp1 is dependent on the receptor CD36 and the src-family tyrosine kinase p59^{fyn}, as Tsp1 cannot block neovascularization in mice that lack CD36 or p59^{fyn} (Jiménez et al., 2000). Additionally, CD36 and p59^{fyn} form a complex upon stimulation with Tsp1. p38MAPK is also phosphorylated upon Tsp1 treatment, downstream of CD36 and p59^{fyn} as p38MAPK activation can be blocked by treatment with either a p59^{fyn} antibody or a CD36 blocking antibody (Jiménez et al., 2000). The end result of this CD36-p59^{fyn}-p38MAPK signalling cascade is the activation of caspase 3 and apoptosis (Jiménez et al., 2000). Importantly, this Tsp1 induction of endothelial cell apoptosis is TGF- β independent (Jiménez et al., 2000). Therefore, Tsp1 can mediate important signalling events in a TGF- β dependent and independent manner.

5.1.3 Tsp1 and cancer

Given that Tsp1 is able to regulate angiogenesis and TGF- β signalling, it is no surprise that Tsp1 has been shown to be involved in cancer. Over-expression of Tsp1 in cultured cancer cell lines reduces their ability to form large, metastatic tumours when implanted into mice (Weinstat-Saslow et al., 1994, Streit et al., 1999). This is consistent with the inhibition of angiogenesis by Tsp1. Tsp1 also recruits macrophages to tumours in mice (Martin-Manso et al., 2008). Conversely, deletion of Tsp1 in mice deficient for p53, which are susceptible to

tumours, results in tumours larger than in mice deficient for p53 alone (Lawler et al., 2001).

A decrease in Tsp1 in tumour cells is associated with tumour progression and poor prognosis (Kodama et al., 2001). This is generally achieved not by mutation or deletion of the Tsp1 gene, but by methylation of the Tsp1 promoter, suppressing Tsp1 transcription (Oue et al., 2003, Rojas et al., 2008, Yang et al., 2003, Isenberg et al., 2009). Also, oncogenic Ras is able to inhibit Tsp1 transcription by stabilising c-Myc (Watnick et al., 2003).

Despite these anti-tumourigenic effects, Tsp1 has also been shown to have a pro-tumourigenic role in certain settings. Tsp1 is able to increase invasion of cells *in vitro*, due to increasing their attachment to extra-cellular matrix (Albo et al., 2000). It has also been shown that Tsp1 is increased in certain types of malignant human tumours (Straume and Akslen, 2003). Like many proteins, the role of Tsp1 in tumourigenesis is complex and multi-dimensional (Kazerounian et al., 2008)

These properties present Tsp1 as an interesting protein that may be involved in scribble mediated cell competition. I will now describe experiments designed to validate the upregulation of Tsp1 in scribble knockdown MDCK cells, and investigate its involvement in the death of scribble knockdown cell surrounded by normal MDCK cells.

5.2.1 Screening of conditioned medium

To identify proteins that are involved in cell competition caused by knockdown of scribble in MDCK cells, conditioned medium was screened. A small proportion of the total protein in cells is secreted, so if there are extra-cellular proteins that mediate cell competition these may be easier to identify than cytosolic or membrane proteins. Cell competition could be mediated by extra-cellular factors secreted from either normal MDCK cells or from scribble-knockdown cells.

To attempt to identify secreted proteins that may mediate cell competition, conditioned culture medium was taken from four different cell cultures:

1. MDCK cells alone, incubated with tetracycline
2. pTR MDCK scribble shRNA cells alone, cultured without tetracycline
3. pTR MDCK scribble shRNA cells alone, cultured with tetracycline.
4. A 1:1 mixed culture of pTR MDCK scribble shRNA: normal MDCK cells incubated with tetracycline.

Each culture condition was seeded at 10×10^6 cells per 15 cm dish (3 dishes were seeded for each condition) and allowed to attach to the bottom of the dish. After attachment and formation of cell-cell contacts tetracycline was added as indicated to knockdown scribble and induce cell competition. After 24 h the medium was changed on all culture dishes to DMEM without FCS, but still supplemented with antibiotics and glutamine. FCS was removed because it contains a very high concentration of proteins that would make it harder to identify any differences in the composition of conditioned medium in the different cell cultures.

After a total of 48 h incubation, the medium was collected from all 3 plates of each culture condition and pooled into a 50 ml Falcon tube. The medium was passed through a $0.45 \mu\text{m}$ filter in order to remove any dead cells in the medium that would block later filtration steps. This gave 45 ml of medium for each culture condition. The cell free conditioned medium was then concentrated by passing it through filters that selectively retain protein but remove water. This

reduced the 45 ml of conditioned medium down to 200 μ l, greatly increasing the concentration of protein (Figure 37). 100 μ l of this concentrated medium was boiled with 50 μ l SDS buffer for 5 minutes at 95°C and run on SDS-PAGE gels before ruby staining to identify protein.

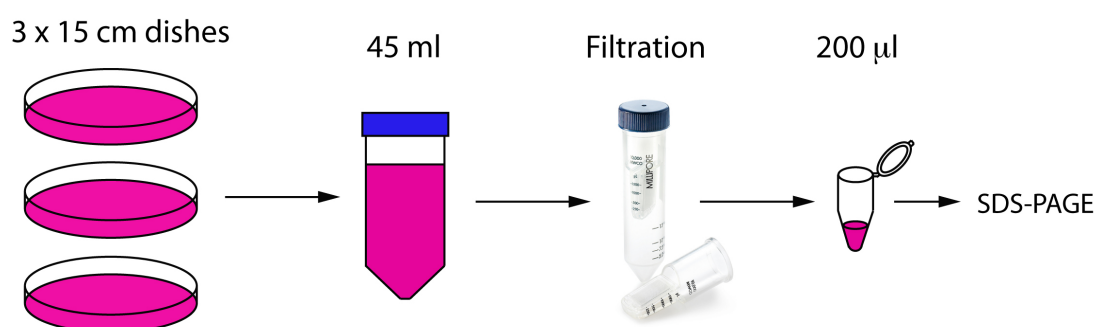


Figure 37 *A summary of the method used to screen conditioned culture medium*

5.2.2 Identification of Thrombospondin 1

When studying the SDS-PAGE gel of conditioned medium from the 4 different cell cultures outlined above, I wished to find bands that were only present in lane 3 (pTR MDCK scribble shRNA plus tetracycline) or lane 4 (1:1 mix of MDCK and pTR MDCK scribble shRNA plus tetracycline). Bands that are only present in lane 3 would represent secreted proteins that are upregulated by the knockdown of scribble. These could be important for communicating the initial signal to the neighbouring cells that the scribble knockdown cells were somehow different. Bands in lane 4 would represent proteins that were only secreted when scribble-knockdown cells were surrounded by normal cells, and these could be key for mediating either the recognition of the scribble knockdown cells or the induction of death in the scribble knockdown cells.

Unfortunately, no bands could be identified that were only present in lane 4. However, a band was repeatedly present in lane 3 (pTR MDCK scribble shRNA alone plus tetracycline) that was also weakly present in lane 4 (Figure 38).

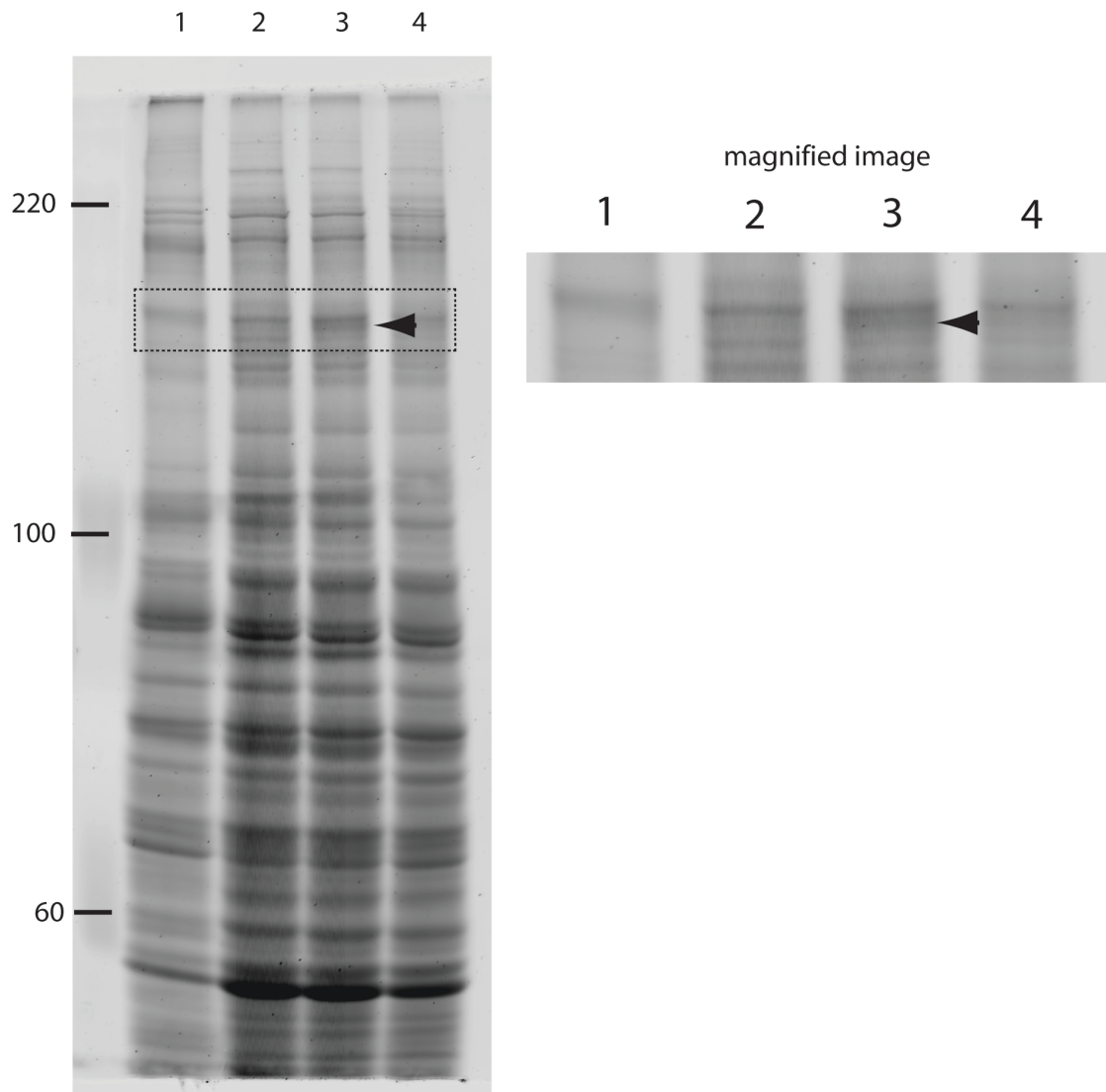


Figure 38 A high molecular weight band is identified that is increased in culture medium from scribble-knockdown MDCK cells. Conditioned medium was taken from MDCK cells alone (1), pTR MDCK scribble shRNA cells without (2) and with tetracycline (3) and 1:1 mixed culture of MDCK: pTR MDCK scribble shRNA cells with tetracycline (4). Samples were run on 8% SDS-PAGE gels and protein visualised with Ruby staining. Dashed box indicates enlarged area.

This band was excised from gel and sent to a regular collaborator of the lab, Dr. Ikegawa of Kyoto Prefecture University Medical School for analysis by mass spectrometry. This analysis identified the band as Thrombospondin 1. Monomeric thrombospondin 1 has a molecular mass of approximately 140 kDa, which broadly fits its localisation on the SDS-PAGE gel (Lawler et al., 1978). It is

also a secreted protein, so it is logical that it can be found in conditioned medium.

5.2.3 Validation of Thrombospondin 1

In order to be certain that the band identified is thrombospondin 1, I ran the samples used for its identification on a SDS-PAGE gel and Western blotted with an anti-Tsp1 antibody.

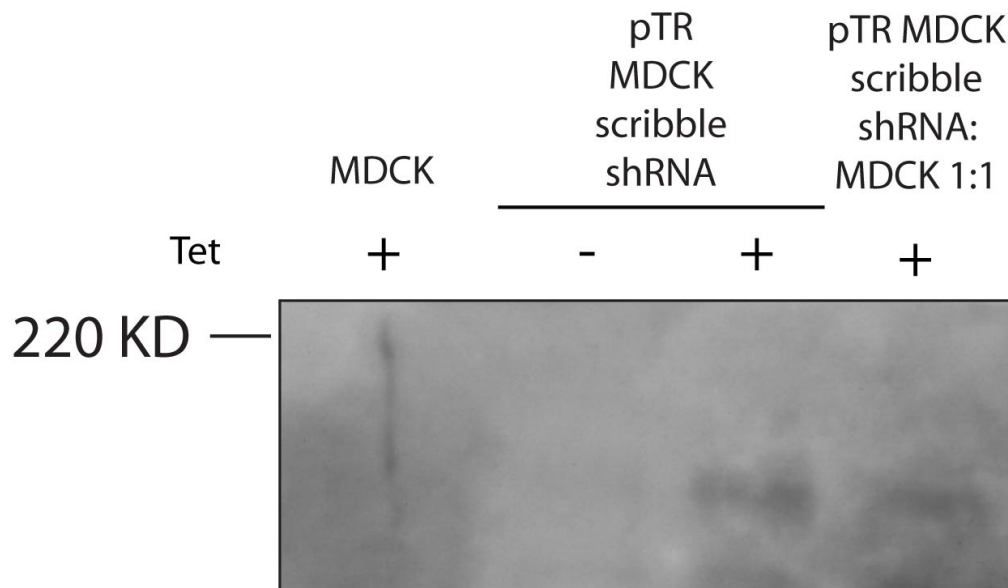


Figure 39 Scribble knockdown increases Tsp1 level in conditioned medium. Conditioned medium taken from MDCK, pTR MDCK scribble shRNA +/- tetracycline and 1:1 pTR MDCK scribble shRNA: normal MDCK cell mixed culture was concentrated and run on an SDS-PAGE gel before Western blotting with an anti-Tsp1 antibody, This validates the mass spectrometry data that knockdown of scribble causes an increase in the level of secreted Tsp1.

Western blotting identified a single band present at approximately 140-170 KDa in conditioned medium taken from pTR MDCK scribble shRNA cells cultured with tetracycline. There was also a weak band in present in the conditioned medium taken from the pTR MDCK scribble shRNA and normal MDCK cell mixed culture, which is presumably due to the presence of scribble-knockdown cells (Figure 39). No Tsp1 was detectable in the conditioned medium taken from pTR MDCK scribble shRNA cells cultured without tetracycline, or MDCK cells cultured with

tetracycline. This shows that the increase in Tsp1 is not specific to the pTR MDCK scribble shRNA cell line or incubation with tetracycline, but is due to knockdown of scribble induced by addition of tetracycline.

5.2.4 Thrombospondin 1 is upregulated at mRNA level

I have described that knockdown of scribble in MDCK cells causes an increase in the level of Tsp1 in conditioned medium. This could be explained by two possibilities. Knockdown of scribble protein and the consequential disruption of cellular morphology (as described in Chapter 3) could cause an increase in Tsp1 secretion from the cell, which would result in more Tsp1 found in conditioned medium. Alternatively, knockdown of scribble could cause an increase in the level of Tsp1 transcription. To identify any effect of scribble knockdown on Tsp1 transcription, levels of Tsp1 mRNA were analysed using quantitative polymerase chain reaction (qPCR).

qPCR is a method used to amplify and quantify the abundance of a target DNA sequence. Primers specific to a target gene of choice are used to amplify a short DNA sequence using standard PCR methodology. Newly made double stranded DNA is bound to a fluorescent label, e.g. SYBR Green, which allows quantitative measurement of the amount of PCR product in real time. In combination with reverse transcription of RNA, qPCR can be used to accurately measure the relative amounts of mRNA transcript for a specific gene.

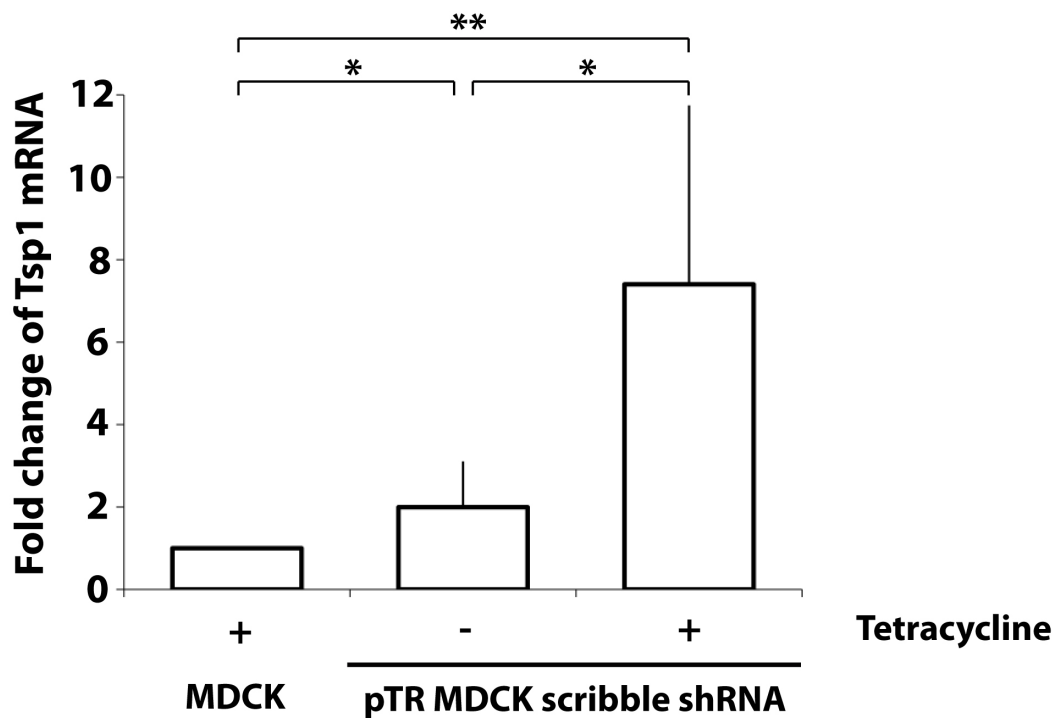


Figure 40 Knockdown of scribble causes an increase in Tsp1 mRNA. The level of Tsp1 mRNA in MDCK cells and pTR MDCK scribble shRNA cells (-/+ 72 h tetracycline) was analysed using qPCR. A clear upregulation of Tsp1 mRNA can be seen when scribble is knocked down. Data is mean of seven independent experiments. Error bars represent one standard deviation. ** $p < 0.005$, * $p < 0.05$.

Tetracycline was added to MDCK cells and one set of pTR MDCK scribble shRNA cells for 72 h. In analysis of the qPCR data, the levels of Tsp1 mRNA in MDCK samples was set as the benchmark, so was always 1. There was a small but consistent increase in the level of Tsp1 transcript in pTR MDCK scribble shRNA cells (1.99) incubated without tetracycline compared to normal MDCK cells treated with tetracycline. There was a mean 7.4 fold increase of Tsp1 transcript in pTR MDCK scribble shRNA cells incubated with tetracycline compared to normal MDCK cells, which was highly statistically significant ($p < 0.005$) (Figure 40). These data support the Western blotting data and show that the increase in Tsp1 protein in conditioned medium taken from scribble-knockdown cells is due to an increase in the level of Tsp1 mRNA in scribble-knockdown cells.

5.2.5 How is Tsp1 mRNA regulated?

How could scribble-knockdown cause an increase in Tsp1 mRNA? The promoter of Tsp1 contains a serum response element (SRE). This means that Tsp1 transcription is upregulated in response to serum stimulation and other external stimuli (Laherty et al., 1989). Downstream of serum in this response are small GTPases (Hill et al., 1995); specifically, it has been shown that Rac1 may regulate serum induced transcription of Tsp1 (Giehl et al., 2008).

In mammalian cells, scribble forms a tight complex with β PIX, a cdc42 and Rac GEF (Audebert et al., 2004). In scribble knockdown cells, although RacGTP levels are not altered (Qin et al., 2005b), the distribution of active Rac is disrupted (Dow et al., 2007). Therefore, it was hypothesised that disruption of the localisation of cellular RacGTP in scribble-knockdown cells may be responsible for increased Tsp1 transcription.

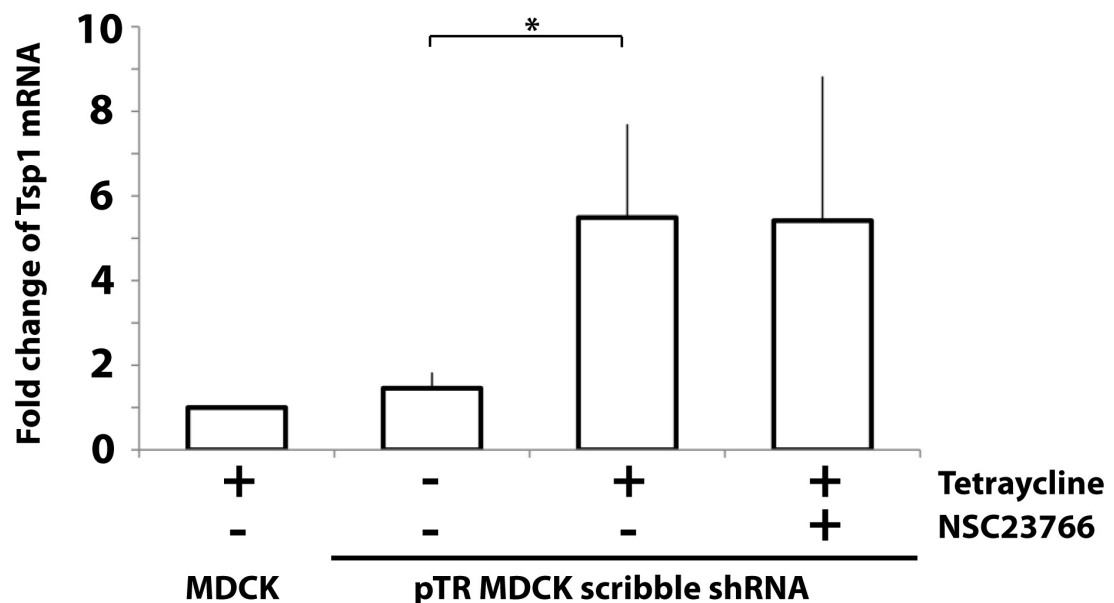


Figure 41 *Rac inhibitor NSC23766 does not block Tsp1 mRNA upregulation in scribble-knockdown cells. Where indicated, tetracycline was added for 72 h and NSC23766 added for 8 h. Data is mean of three independent experiments. Error bars are one standard deviation. * $p < 0.05$ Student's T-test.*

MDCK and pTR MDCK scribble shRNA cells were incubated with and without tetracycline for 72 h. 100 μ M NSC23766, an inhibitor of Rac1 activation (Gao et al., 2004), was added to scribble-knockdown cells for the final 8 h of incubation. The level of Tsp1 mRNA was very similar in scribble-knockdown cells incubated with or without the Rac1 inhibitor NSC23766 (Figure 41). Therefore, increase of Tsp1 transcription in scribble-knockdown MDCK cells is not dependent on Rac activity.

5.2.6 Scribble knockdown cells have increased phospho-SMAD2

As described in section 5.2.1, one of the classic functions of Tsp1 is to activate latent TGF- β . To ascertain if the increase in Tsp1 in scribble knockdown cells has biological consequences, the level of phosphorylated SMAD2 was checked by Western blotting. Upon binding of TGF- β to the TGF- β receptor I and II, TGF- β receptor I phosphorylates SMAD2 at serines 465/467 (Shi and Massagué, 2003). Phosphorylated SMAD2 then complexes with SMAD4 and translocates to the nucleus where the transcription of target genes is initiated with the help of co-factors (Shi and Massagué, 2003). Phosphorylation of SMAD2, therefore, is an early event in the activation of TGF- β signalling and an excellent indicator that the TGF- β signalling pathway is activated.

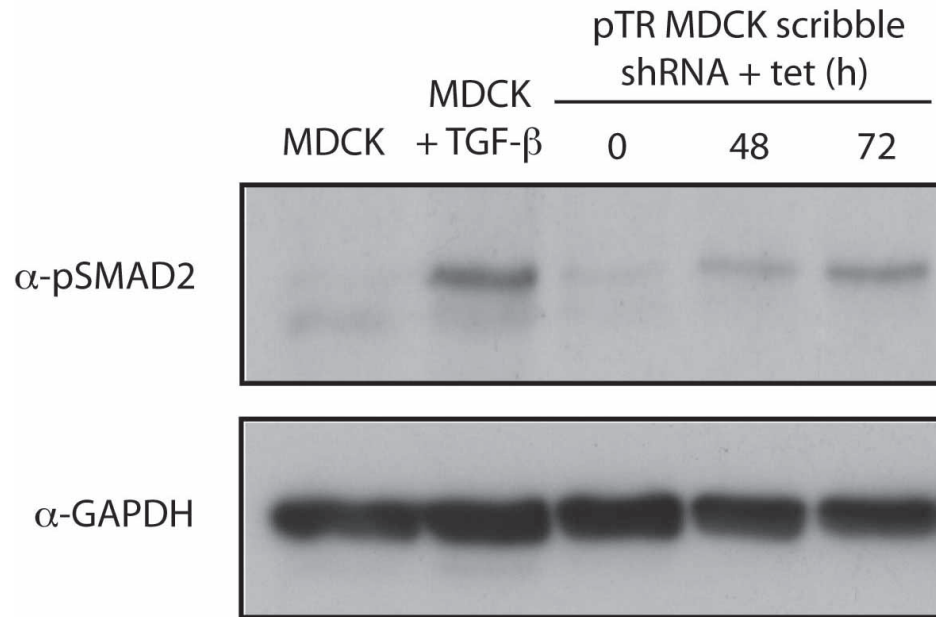


Figure 42 Scribble knockdown cells have increased phosphorylated SMAD2. MDCK cells were incubated with tetracycline for 72h (MDCK) or 2 μ g/ml recombinant TGF- β for 2h (MDCK + TGF- β). pTR MDCK scribble shRNA cells were incubated with tetracycline for 0, 48 or 72h before cell lysates were taken. Samples were probed with antibodies against phosphorylated SMAD2 to test for activation of TGF- β signalling and GAPDH as a loading control. Increased incubation of pTR MDCK scribble shRNA cells with tetracycline results in an increase in the level of phosphorylated SMAD2.

pTR MDCK scribble shRNA cells were incubated with tetracycline for 0, 48 and 72 hours to induce different levels of scribble knockdown. MDCK cells were incubated with tetracycline for 72 h or 2 μ g/ml recombinant human TGF- β (Peprotech, NJ) for 2 h. Recombinant TGF- β was added to ensure that MDCK cells can respond to TGF- β .

MDCK cells incubated with just tetracycline as control had very low levels of phosphorylated SMAD2 (pSMAD2) (Figure 42). MDCK cells incubated with TGF- β showed significant levels of pSMAD2, demonstrating that MDCK cells are able to respond to TGF- β (Figure 42). pTR MDCK scribble shRNA cells incubated without tetracycline had a very low level of pSMAD2, comparable to MDCK cells incubated with just tetracycline. Substantial pSMAD2 could be seen when pTR

MDCK scribble shRNA cells were incubated with tetracycline for 48h and a higher level still could be seen in pTR MDCK scribble shRNA cells incubated with tetracycline for 72h (Figure 42). This suggests that the increase in Tsp1 protein in scribble-knockdown cells is activating latent TGF- β , which then activates TGF- β signalling in scribble-knockdown cells.

5.2.7 SMAD4 localisation in scribble-KD cells

Scribble-knockdown MDCK cells up-regulate Tsp1 transcription and have increased phosphorylation of SMAD2, evidence that they have activated TGF- β signalling. If the increase in TGF- β signalling is important for the death of scribble-knockdown MDCK cells when surrounded by normal MDCK cells, then TGF- β signalling must also be activated in this context. To address this question, I studied the localisation of SMAD4 in scribble knockdown-cells surrounded by normal cells. As described above, upon TGF- β receptor stimulation phosphorylated SMAD2 complexes with SMAD4 and translocates to the nucleus. Therefore, an increase in SMAD4 nuclear localisation is an indication that there is increased TGF- β signalling in the cell.

pTR MDCK scribble shRNA cells were stained with green CMFDA dye, mixed 1:10 with normal MDCK cells and seeded onto glass coverslips. Tetracycline was added for 48 h before methanol fixation. Cells were stained with an antibody against SMAD4 and analysed with confocal microscopy. Consistent with Western blotting data described above, MDCK cells showed little nuclear localisation of SMAD4 (Figure 43). In some cases, scribble-knockdown cells could be seen that had high levels of SMAD4 in the nucleus (Figure 43). This indicates that TGF- β signalling is activated in scribble-knockdown cells that are surrounded by normal cells, as well as well cultured alone.

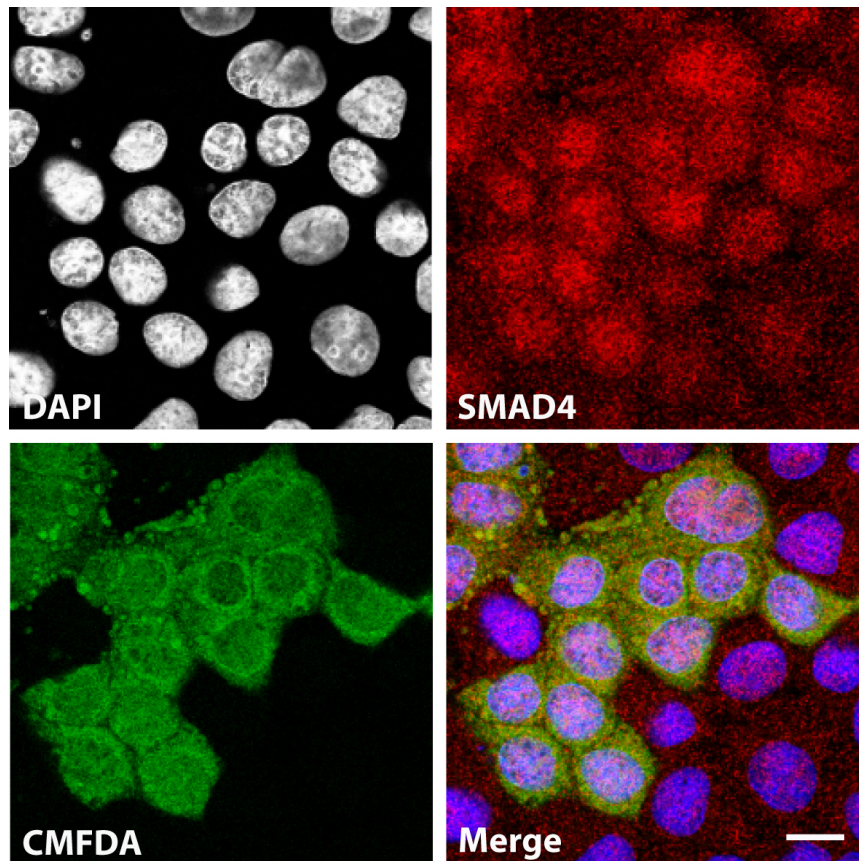


Figure 43 Increased SMAD4 nuclear translocation in scribble knockdown cells surrounded by normal MDCK cells. Green CMFDA pTR MDCK scribble shRNA cells were mixed with normal MDCK cells, seeded onto glass coverslips and incubated with tetracycline for 48 h. Cells were fixed with methanol and immunostained with SMAD4. Increased nuclear localisation of SMAD4 can be seen in scribble-knockdown cells. Scale bar represents 10 μ m.

This phenotype was observed inconsistently. This could be due to the fact that cells with high TGF- β signalling are rapidly eliminated from the epithelium as it may only occur in cells that have very low levels of scribble protein, as indicated by the data showing that TGF- β signalling in scribble-knockdown cells is highest after 72 h incubation with tetracycline. Alternatively, this could be a technical limitation of the antibody or the fixation protocol.

5.2.8 Scribble mutant clones in *D. melanogaster* do not up-regulate Thrombospondin

Scribble was originally identified in *D. melanogaster* and has been well studied in the fly (Bilder and Perrimon, 2000, Bilder et al., 2003, Bilder et al., 2000b, Zeitler et al., 2004). To study the evolutionary conservation of upregulation of Tsp1 in scribble-knockdown cells, the level of *D. melanogaster* Thrombospondin was analysed in *scribble* mutant clones.

In order to check the anti-TSP antibody works and that TSP could be identified in *D. melanogaster*, clones of cell over-expressing TSP were created in wing imaginal discs. As expected, clones expressing UAS-TSP in third instar larval wing imaginal discs showed very high levels of staining with the anti-TSP antibody (Figure 44). No TSP staining could be seen in the wing disc outside of the UAS-TSP clones, consistent with previous reports (Butler et al., 2003).

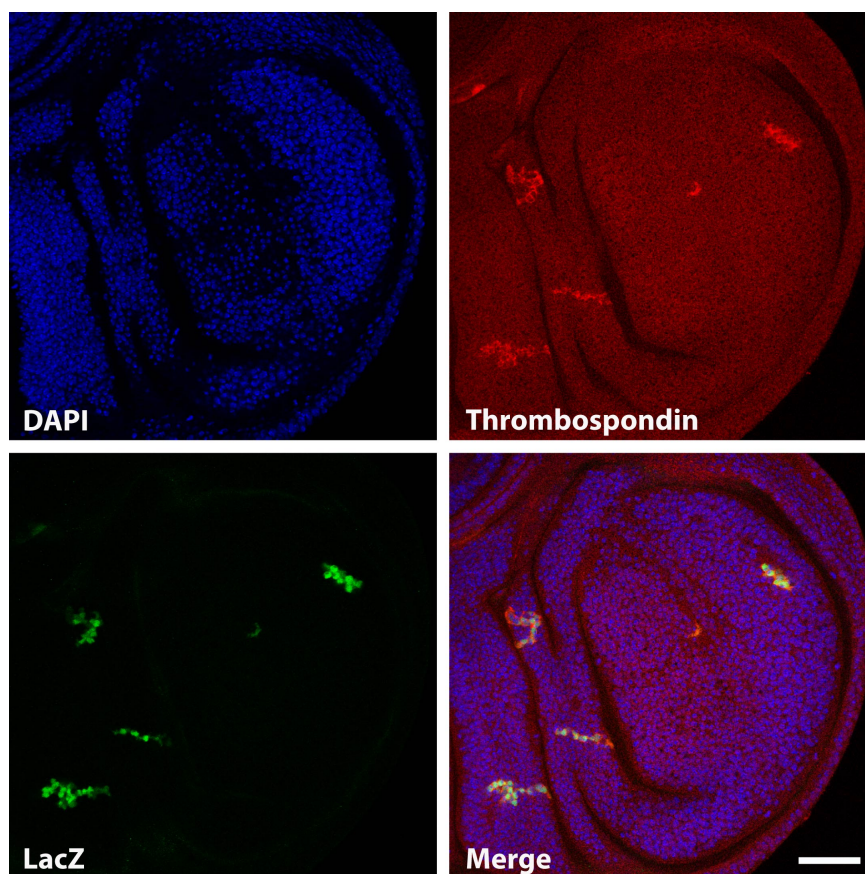


Figure 44 Anti-TSP antibody can recognise TSP produced in the wing disc. Clones expressing UAS-TSP were induced in wing imaginal discs of *D. melanogaster*

larvae. Clones were marked by expression of LacZ (bottom left panel). The anti-TSP antibody used clearly detects TSP expressed in these clones. Scale bar is 40 μ m.

Scribble mutant clones were induced by heatshock and larvae were dissected 48h later, when the larvae were in the third instar of larval development. Wing discs were stained with anti-TSP antibody to visualise TSP production in *scribble* mutant clones.

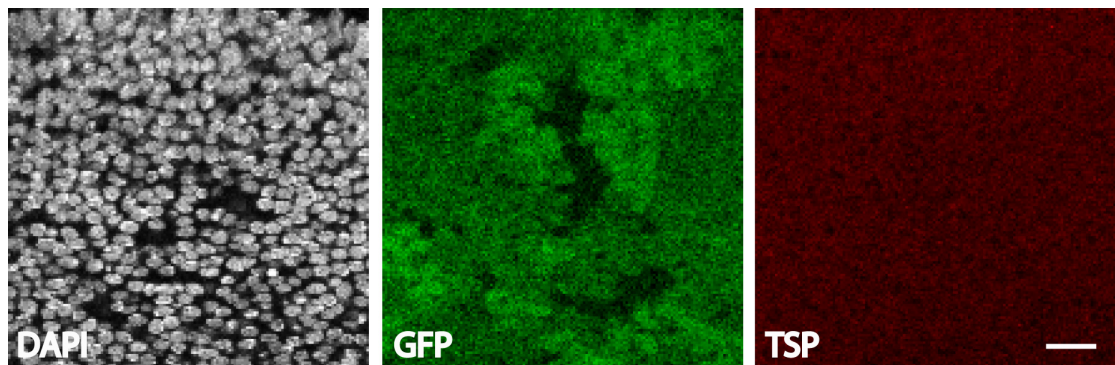


Figure 45 *Scribble* mutant clones in *Drosophila* do not up-regulate TSP. The level of TSP protein in *scribble* mutant clones in third instar larvae was analysed with an anti-TSP antibody. Twin spot clones were generated 48h prior to dissection of larvae. Black cells are *scribble*^{-/-}, bright green cells are *scribble*^{+/+} and middle green cells are *scribble*^{+/-}. Scale bar is 10 μ m.

Staining *scribble* clones with an anti-TSP antibody showed no evidence that TSP is up-regulated in these clones (Figure 45). Multiple wing discs were examined and in all there was no difference in TSP staining between *scribble* mutant cells and wild-type cells..

5.2.9 *Scribble* mutant clones in *Drosophila melanogaster* do not have increased pMad

I have described that knockdown of *scribble* in MDCK cells results in an increase of Tsp1 transcription and an increase in TGF- β signalling, as shown by an increase in pSMAD2 levels (Figure 42). Although there is no up-regulation of TSP

in *D. melanogaster*, TGF- β signalling may be activated in these cells by another mechanism. The TGF- β superfamily is well conserved in *D. melanogaster* and the basic ligand-receptor-receptor SMAD system is preserved (Raftery and Sutherland, 1999). The most well studied member of the family is Decapentaplegic (Dpp), which is an orthologue of mammalian BMP2/4 (Raftery and Sutherland, 1999, Padgett et al., 1993). Dpp is critical for many morphological processes in *D. melanogaster* development, most notably in the wing imaginal disc, in which it has been extensively studied (Affolter and Basler, 2007). Dpp binding to the receptor thickvein phosphorylates Mothers against decapentaplegic (MAD). Phosphorylation of MAD is therefore a good measure of TGF- β superfamily signalling in *D. melanogaster*.

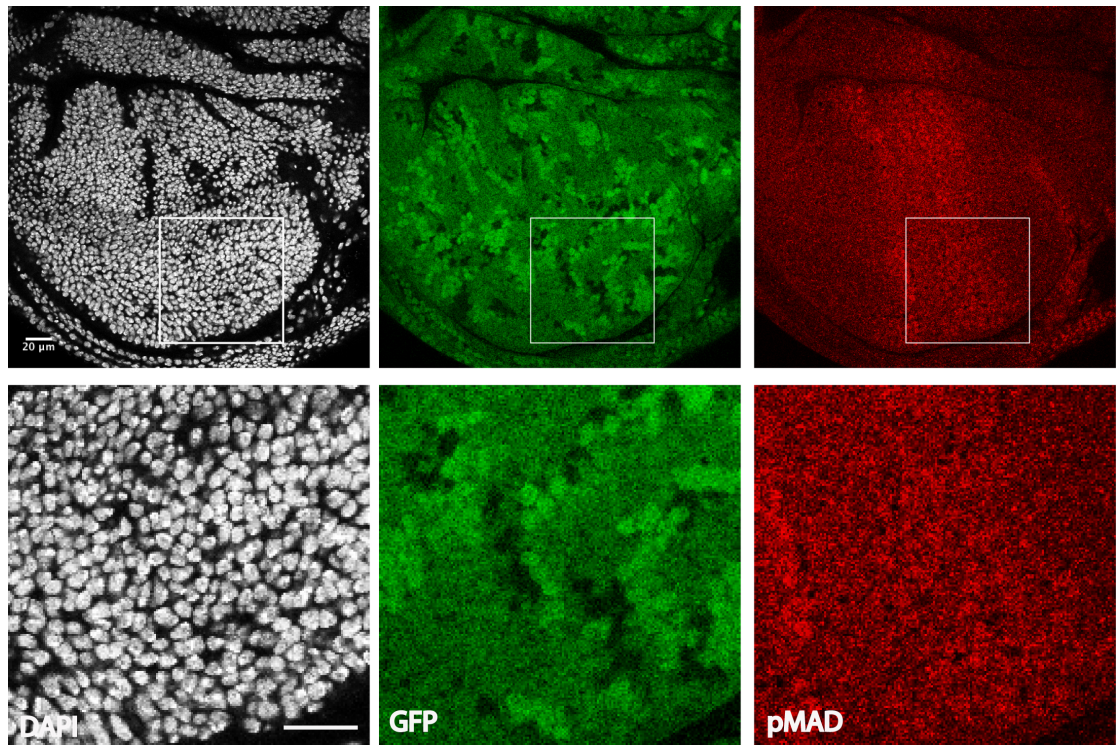


Figure 46 *D. melanogaster* scribble mutant clones do not have increased pMAD. Scribble mutant clones were induced in larval wing discs and larvae dissected at third instar stage. Black cells are scribble $^{-/-}$, bright green cells are scribble $^{+/+}$ and middle green cells are scribble $^{+/-}$. Areas outlined in the top row of images are enlarged in the bottom row. No upregulation of pMAD can be seen in scribble $^{-/-}$ clones. The typical wing disc pattern of pMAD at the anterior-posterior boundary can be seen. Scale bar is 20 μ m.

Analysis of *scribble* mutant clones demonstrates that there is no increase in pMAD (Figure 46). pMAD was seen in a central stripe down the centre of the wing disc, along the anterior-posterior boundary as expected (Teleman and Cohen, 2000). No increase in pMAD could be seen in *scribble* mutant clones either in areas of high pMAD intensity or in areas of low pMAD intensity (Figure 46). So at least in wing discs, loss of *scribble* does not result in an increase in TGF- β signalling.

5.2.10 Blocking TGF- β receptor activity does not inhibit death of *scribble* knockdown cells

Data so far presented in this chapter has mostly concerned *scribble*-knockdown cells on their own. The central theme of this thesis is that loss of *scribble* in mammalian epithelial cells causes cell competition. It is, therefore, critical to determine what role the upregulation of Tsp1 and activation of TGF- β signalling has in the death of *scribble*-knockdown MDCK cells when surrounded by normal MDCK cells.

Experiments in *D. melanogaster* have shown that apoptosis is induced when two populations with different levels of TGF- β /Dpp signalling meet (Adachi-Yamada et al., 1999, Adachi-Yamada and O'Connor, 2002, Manjón et al., 2007). This work suggests that, at least in *D. melanogaster*, there exists a mechanism by which cells are able to recognise different levels of Dpp signalling and apoptosis occurs as a result. Could a similar mechanism occur in cultured mammalian cells that is important for the death of *scribble*-KD cells? To address this question, time-lapse experiments were setup as described in Figure 12 with the addition of 2 μ M SD-208, an inhibitor of the kinase domain of TGF- β receptor I (Uhl et al., 2004).

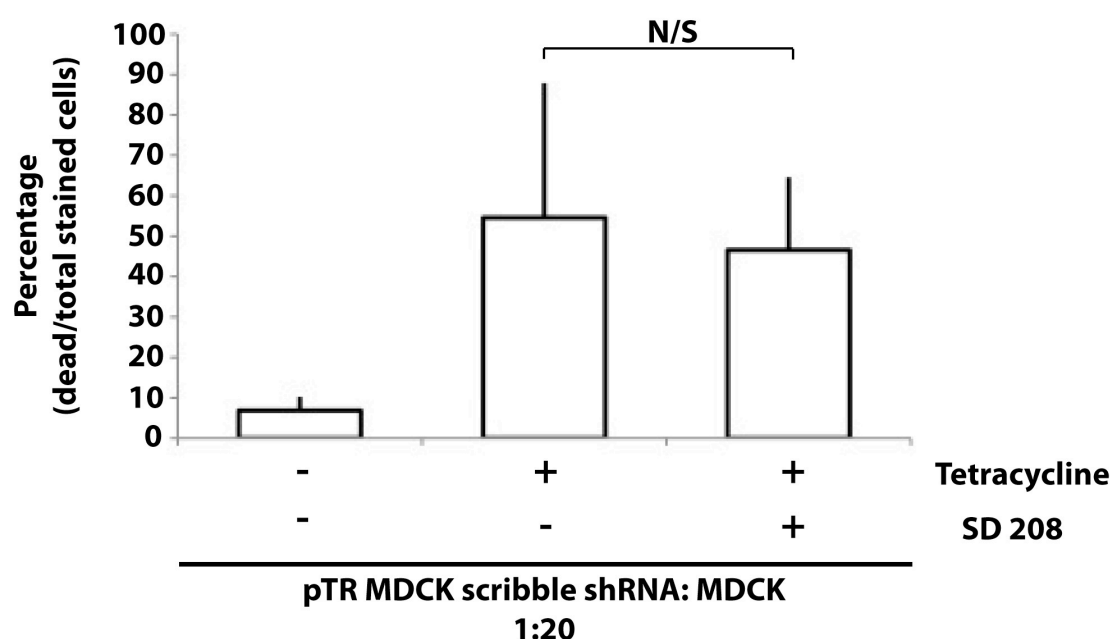


Figure 47 *SD-208 does not inhibit death of scribble-knockdown cells surrounded by normal MDCK cells.* Green CMFDA labelled pTR MDCK scribble shRNA cells were mixed with normal MDCK cells and observed using time-lapse microscopy +/- tetracycline and +/- 2 μ M SD-208. Addition of SD-208 did not significantly suppress death of scribble-KD cells. Data is mean of 3 independent experiments. Error bars represent one standard deviation. N/S = no significant difference, Student's T-test.

As expected, incubation of mixed cultures without tetracycline resulted in very low levels of cell death in stained pTR MDCK scribble shRNA cells. Addition of tetracycline resulted in over 50% of stained scribble-knockdown cells dying when surrounded by normal MDCK cells, similar to previous experiments (Figure 14). Addition of the TGF- β receptor I inhibitor SD-208 had no effect on cell death of scribble-knockdown cells surrounded by normal MDCK cells (Figure 47). This demonstrates that inhibition of TGF- β signalling alone is not sufficient to block cell competition caused by the knockdown of scribble.

5.3 Discussion

5.3.1 Knockdown of scribble up-regulates Tsp1

I have shown that knockdown of scribble in MDCK increases the amount of Tsp1 mRNA and secreted Tsp1. To my knowledge, a relationship between scribble and Tsp1 has not previously been reported. Proteins related to both scribble and Tsp1 have previously been genetically linked; it has been shown in *D. melanogaster* that Lgl and semaphorin 5c, which shares homology with mammalian Tsp1, are genetically linked (Woodhouse et al., 2003).

It is not known how knockdown of scribble results in upregulation of Tsp1. The promoter of Tsp1 contains a serum response element (SRE) (Laherty et al., 1989) which is induced by small GTPases downstream of serum and other stimuli (Hill et al., 1995). In particular, the small GTPase Rac has been shown to influence Tsp1 transcription (Giehl et al., 2008). It has previously been shown that active, GTP bound Rac is mislocalised in scribble-knockdown epithelial cells (Dow et al., 2007). It was theorised that knockdown of scribble could free β PIX and lead to mislocalisation of RacGTP in scribble-knockdown MDCK cells, which may activate transcription from the SRE. Attempts to block increase of Tsp1 mRNA caused by scribble-knockdown with NSC23766, an inhibitor of Rac1 activation, were unsuccessful. This may be due to involvement of other small GTPases in activation of Tsp1 transcription, or poor affinity of the inhibitor for β PIX, the Rac/cdc42 GEF that associates with scribble (the affinity of NSC23766 has only been tested for a limited set of RacGEFs (Gao et al., 2004)). It should be noted that the original identification of Tsp1 upregulation in scribble knockdown cells was in cells cultured in serum free medium. Therefore, it seems unlikely that an increased response to serum is responsible for the observed upregulation of Tsp1 mRNA in scribble-knockdown cells.

Tsp1 has also been shown to be regulated by the proto-oncoprotein Myc (Watnick et al., 2003). Myc represses transcription of Tsp1; could myc activity be lower in scribble knockdown cells and consequently repression of Tsp1

transcription is lower? In *D. melanogaster* it has been shown that clones of *lgl*^{-/-} mutant cells have lower dMyc expression than surrounding wild type cells, although no such relationship has been reported with myc and scribble (Froldi et al., 2010). Scribble and myc have been shown in mammalian cells to co-operate in promoting a more transformed phenotype, but myc activity in scribble knockdown cells was not addressed in this study (Zhan et al., 2008). It would be interesting to study the level of myc activity in the scribble-knockdown MDCK cells used in this study. As well as possibly explaining the increase in Tsp1 transcription in scribble-knockdown MDCK cells, this may also shed light on the cell competition phenotype of scribble-knockdown cells. It has been widely observed in *D. melanogaster* that cells with lower dMyc expression are removed by cells expressing a higher level of dMyc (de la Cova et al., 2004, Moreno and Basler, 2004). To study this possible relationship between scribble and myc in MDCK cells, a cell line has been made containing inducible scribble shRNA and c-myc GFP. Time constraints meant that I was unable to study this cell line before completion of this thesis. Due to published data linking myc and scribble, I believe this would be an interesting future avenue of research with respect to the cell death of scribble-knockdown cells surrounded by normal MDCK cells, and also with respect to the upregulation of Tsp1 in scribble-knockdown MDCK cells (Zhan et al., 2008, Froldi et al., 2010, Watnick et al., 2003).

5.3.2 Possible implications of Tsp1 on the death of scribble-knockdown cells surrounded by normal MDCK cells

One of the principal biological functions of Tsp1 is the induction of apoptosis in blood vessels; this is responsible for the anti-angiogenic function of Tsp1 (Jiménez et al., 2000). This function involves activation of p38MAPK, which I have shown is elevated in scribble-knockdown cells surrounded by normal MDCK cells, and is required, at least in part, for the induction of cell death in these scribble-knockdown cells (Figure 33-35).

Tsp1 is up-regulated in scribble-knockdown MDCK cells in a cell-autonomous manner, whereas the up-regulation of p38MAPK activity only occurs when scribble-knockdown cells are surrounded by normal MDCK cells. Therefore, it is unlikely that Tsp1 is entirely responsible for the increase in p38MAPK activity. It is possible that other proteins involved in the activation of p38MAPK by Tsp1 are increased only in scribble-knockdown cells surrounded by normal MDCK cells. I attempted to study the level of CD36, which is essential for Tsp1 activation of p38MAPK in blood vessels, but could not detect any staining by immunofluorescence (Jiménez et al., 2000).

Tsp1 is known to inhibit the response of cells to stress caused by reactive oxygen species by inhibiting nitric oxide signalling (Isenberg et al., 2006, Wink et al., 1993, Isenberg et al., 2009). Reactive oxygen species are known to be second messengers in a number of apoptotic signalling pathways, as well as inducing apoptosis themselves when highly abundant (Ott et al., 2007). It has been shown that Tsp1 and the receptor CD47 are required for the induction of apoptosis in cells in response to radiation (Maxhimer et al., 2009). It is possible the high expression level of Tsp1 in scribble-knockdown MDCK cells makes them susceptible to a stress caused by being surrounded by normal MDCK cells. This would make Tsp1 downstream of another stress inducing signal so would not explain why scribble-knockdown cells die when surrounded by normal MDCK cells. This is a very hypothetical explanation of the role of Tsp1 in the death of scribble-knockdown MDCK cells when surrounded by normal MDCK cells and needs to be studied further, which would require knocking down Tsp1 in scribble-knockdown MDCK cells.

5.3.3 Implications for scribble knockdown mediated Tsp1 up-regulation in tumourigenesis

Tsp1 has long been known to be an inhibitor of tumour progression, through two main mechanisms (Miao et al., 2001). Tsp1 can activate latent TGF- β , which itself has many properties relating to tumourigenesis (discussed below). Tsp1 can also

inhibit tumour progression due to inhibition of angiogenesis, which is apoptosis dependent and TGF- β independent (Streit et al., 1999, Jiménez et al., 2000). Angiogenesis is an essential step in tumour progression; as tumours enlarge they outgrow the natural blood supply and must stimulate new blood vessel formation in order to supply the tumour with nutrients. The formation of new blood vessels in a tumour is a sign of tumour progression and is termed the angiogenic switch (Bergers and Benjamin, 2003). Inhibition of this step of tumourigenesis is, therefore, a powerful action of a tumour suppressor. To counteract these inhibitory effects, the promoter of Tsp1 is often methylated in tumours to prevent transcription of Tsp1, among other strategies (Rojas et al., 2008, Yang et al., 2003, Oue et al., 2003, Filleur et al., 2001).

It is counterintuitive, then, that loss of a tumour suppressor should increase transcription of an anti-angiogenic factor. It would seem that up-regulation of Tsp1 when scribble is lost could be a tumour suppressive response. Several oncogenes have been reported to regulate Tsp1 transcription. Oncogenic Ras stabilises c-myc, and c-myc is able to suppresses Tsp1 transcription (Watnick et al., 2003). Ras or myc are over-expressed in a huge number of cancers, so this is likely to be a common mechanism to inhibit Tsp1 transcription. However, the role of Tsp1 in tumours is complicated and may, in some situations, be pro-tumourigenic. Expression arrays of thyroid carcinomas with BRAFV600E mutations have uncovered a possible pro-tumourigenic, pro-metastatic role for Tsp1 in these tumours (Nucera et al., 2010). These contrasting reports demonstrate that Tsp1 can have very different effects on tumours, depending on the genetic background. Therefore, any effects of scribble-knockdown induced Tsp1 expression would be equally variable and dependent on other mutations in the tumour cells.

5.3.4 TSP and pMAD are not upregulated in *D. melanogaster scribble* mutant clones

I have shown that there is no upregulation of either *D. melanogaster* thrombospondin or the *D. melanogaster* TGF- β pathway in clones of scribble cells in *D. melanogaster* wing imaginal discs. Woodhouse et al show that pMAD is increased in *lgl*^{-/-} mutant brain tissue, and that this is dependent on semaphorin 5c (Woodhouse et al., 2003). Semaphorin 5c contains type I thrombospondin repeats; these are the domains present in Tsp1 that are responsible for activation of latent TGF- β (Schultz-Cherry et al., 1995). This is very similar to data reported in this thesis, where loss of scribble in MDCK cells increases Tsp1 transcription and SMAD2 phosphorylation. These data suggest that the mechanism of scribble loss, upregulation of thrombospondin type I repeat containing proteins and TGF- β activation may be conserved in *D. melanogaster*. Clonal analysis of *scribble* mutant cells may be an unsuitable method to study this pathway; it has not previously been reported that *lgl*^{-/-} clones up-regulate pMAD in wing imaginal discs, despite these data shown by Woodhouse and colleagues. It would be very interesting to analyse the level of pMAD in *scribble* mutant brain tissue using the same technique as Woodhouse et al, as this may reveal a general up-regulation of pMAD in brain tissue whole mutant for any member of the Scribble complex. However, if this pathway was conserved then it would be unlikely to be important for cell competition as increased levels of pMAD cannot be seen in outcompeted *scribble* clones.

5.3.5 Scribble knockdown cells have activated TGF- β signalling

I have shown that scribble-knockdown MDCK cells have increased TGF- β signalling as evidenced by increased levels of phosphorylated SMAD2. This is the first time that scribble has been implicated in the TGF- β signalling pathway. It is likely that the increase in TGF- β signalling is due to extra-cellular activation of latent TGF- β by Tsp1, as this is a very well characterised function of Tsp1 (Lawler, 2000). However, attempts to block the activation of TGF- β signalling in scribble knockdown cells with short peptides that block the interaction between Tsp1 and TGF- β LAP were unsuccessful (Ribeiro et al., 1999). This could be due

to poor penetration of the peptide to the basolateral surface where Tsp1 is secreted (Prabakaran et al., 1999). MDCK cells form a tight epithelial barrier and this may have prevented the peptides, added to medium present on the apical surface, from accessing Tsp1.

It is very interesting that loss of scribble can activate TGF- β signalling. TGF- β is highly conserved throughout evolution and plays a role in many biological and developmental processes and can activate transcription of an enormous variety of genes (Kitisin et al., 2007, Shi and Massagué, 2003). Given this diversity of function, it is not surprising that the role of TGF- β in cancer is complex. Activation of TGF- β signalling can aid or hinder tumour progression, depending the stage of tumourigenesis (Massagué, 2008). In early stages of tumourigenesis, TGF- β activity represses tumour progression (Massagué, 2008) (Biswas et al., 2004). This repression of tumour progression is achieved by apoptotic or cytostatic means, although the particular molecular mechanism can vary depending on cell type (Siegel and Massague, 2003). In later stages of tumourigenesis, TGF- β signalling is pro-tumourigenic. Vital to this is the ability of TGF- β to induce the epithelial to mesenchymal transition (EMT), which allows tumour cells to metastasise and colonise new tissues (Padua and Massague, 2009).

If scribble knockdown up-regulates TGF- β signalling, it would be expected that they have similar functions in tumourigenesis. The profile of scribble in tumourigenesis is nowhere near as well defined as that of TGF- β . I have shown in this thesis, and others have described previously in *D. melanogaster* (Brumby and Richardson, 2003), that small numbers of scribble-knockdown cells are removed from the epithelium. This is proposed to be a model of the earliest stage of tumourigenesis. I have shown that TGF- β signalling is not essential for this process; indeed, scribble-knockdown cells alone have activated TGF- β signalling and apoptosis is not significantly elevated in these cells. What is more interesting is that scribble has been shown to have a role in metastasis. One of the first properties attributed to scribble was the ability of scribble deletion to co-operate

with RasV12 over-expression to make tumour cells invade other tissues (Pagliarini, 2003). It has also been described that knockdown of scribble in mammalian cells increases cell motility (Qin et al., 2005b) and can co-operate with other mutations to give an invasive phenotype (Dow et al., 2008). There is, therefore, some weak phenotypic correlation between TGF- β activation and scribble knockdown, but the direct effect of TGF- β on scribble phenotypes has not been tested; it would be interesting to examine a possible role of TGF- β in these invasive phenotypes, particularly in mammalian cells.

5.3.6 TGF- β and cell competition

I have shown that despite the increase of TGF- β activity seen in scribble-knockdown MDCK cells alone and when mixed with normal MDCK cells, TGF- β signalling is not required for the induction of cell death in scribble knockdown cell surrounded by normal MDCK cells. It has previously been shown that sharp boundaries of Dpp signalling, a *D. melanogaster* TGF- β superfamily member, can induce cell death (Adachi-Yamada et al., 1999, Adachi-Yamada and O'Connor, 2002). Some of these studies use very high levels of Dpp expression caused by over-expression, but it has also been shown that more modest, naturally induced boundaries of Dpp signalling are also able to induce cell death (Manjón et al., 2007). To my knowledge, induction of cell death due to sharp boundaries of TGF- β signalling has not been shown in mammalian cells. In these studies in *D. melanogaster*, most frequently the cell with lower Dpp signalling is eliminated, although cell death in cells with higher Dpp signalling is also seen (Adachi-Yamada and O'Connor, 2002). Important to note is that cell death is normally seen on either side of the boundary, i.e. in the cells with altered Dpp/TGF- β activity and also the neighbouring wild type cells. This agrees with the hypothesis that this induction of cell death is to restore the normal Dpp signalling gradient (Adachi-Yamada and O'Connor, 2002). Cell death was not frequently seen in normal MDCK cells that surround scribble-knockdown MDCK cells. These data show that it is unlikely that TGF- β is involved in the induction of cell death in scribble-knockdown MDCK cells surrounded by normal MDCK cells.

The failure of SD208 to block death of scribble-knockdown cells surrounded by normal MDCK cells shows that TGF- β signalling is not essential for the death of the scribble-knockdown cells. That inhibition of TGF- β alone does not stop cell competition mediated death of scribble knockdown cells is not necessarily surprising, as no increase in Dpp signalling was detectable in *scribble* mutant clones in *D. melanogaster*; therefore, there must be a TGF- β independent signal that removes cells lacking scribble from mammalian and *D. melanogaster* epithelia.

5.3.7 Conclusion and future outlook

Scribble-knockdown MDCK cells autonomously over-express Tsp1. This increases the level of TGF- β activation in scribble-knockdown cells. It was hypothesised that this could potentially be important for the interaction of scribble-knockdown MDCK cells with normal MDCK cells, but inhibition of TGF- β activity does not inhibit cell death in scribble-knockdown MDCK cells surrounded by normal MDCK cells. This rules out TGF- β as an important pathway in the induction of cell death in scribble-knockdown MDCK cells surrounded by normal MDCK cells.

The complicated range of biochemical pathways Tsp1 is involved in make it difficult to confirm a role of Tsp1 in the death of scribble-KD cells. The only way to properly address this would be to make a cell line that contains anti scribble shRNA and anti-Tsp1 shRNA. This is very technically demanding, in part due to the secreted nature of Tsp1 in MDCK cells. To my knowledge, a MDCK cell line stably expressing two shRNAs has never before been made. An indirect method may be possible, such as over-expression of a regulator of Tsp1 expression such as myc or inhibition of small GTPases that are involved in Tsp1 regulation. These approaches present their own difficulties and will have many effects on MDCK cells outside of that on Tsp1. Disappointingly, it may be impossible to further

investigate the role of Tsp1 in scribble-knockdown mediated cell competition in MDCK cells using methods used in this thesis.

5.3.8 Limitations of the screening approach used and alternative methods

In this chapter I screened concentrated conditioned medium using SDS-PAGE gel electrophoresis. This found Tsp1, an interesting protein that is upregulated due to loss of scribble, but failed to find any proteins specifically increased due to the interaction of scribble-KD cells with normal MDCK cells. There are several problems with this screening approach. Running gels in one-dimension gives poor separation of proteins. Two-dimensional gels are a method to give better separation of proteins; this was attempted, but did not give a reproducible enough pattern to allow identification of proteins increased only when scribble-KD cells interact with normal MDCK cells. In addition, screening conditioned medium only allows identification of extra-cellular proteins and there is little evidence that cell competition is mediated by long range diffusible proteins rather than local interactions (Senoo-Matsuda and Johnston, 2007).

Whilst I was completing my PhD, other members of the Fujita lab established techniques such as biotinylation of cell surface proteins, which allows labelling and purification of cell surface proteins (Jang and Hanash, 2003). This is likely to be a more suitable method to identify proteins that are specifically enriched at the interface between scribble-KD cells and normal MDCK cells. An alternative method is stable isotope labelling with amino acids in cell culture (SILAC), a method that has been setup by collaboration with Dr. Jorgensen (Institute for Cancer Research, London). SILAC uses isotopically labelled amino acids to label different cell populations and can be used to identify proteins that are modified or transcribed only when in mixed culture (Jorgensen et al., 2009). This is an excellent method for identifying proteins that are involved in the interaction between two cell types and would be interesting to apply to the interaction between scribble-KD cells and normal cells. Another positive of this technique is that cell lysates are screened by mass spectrometry, so post-translational

modifications can be identified (Jorgensen et al., 2009). However, currently cells can only be grown in mixed culture for less than ten hours which is much shorter than the time periods used in this thesis (64 h) and may present technical difficulties. If time had allowed, I would have liked to employ advanced proteomic approaches such as these in order to try and find proteins that are specifically upregulated by the interaction of scribble-KD and normal MDCK cells.

Chapter 6 Final Discussion

Chapter 6 Final Discussion

6.1 Summary of data presented

In this thesis, I have shown that knockdown of scribble in MDCK cells causes mild defects in epithelial morphology. The major finding of this study is that scribble-knockdown MDCK cells die when they are surrounded by normal MDCK cells. Cell death can occur independently of apical extrusion. p38 activity is increased in scribble-KD cells and inhibition of p38 activity significantly suppresses death of scribble-KD cells. Knockdown of scribble in MDCK cells increases transcription of Tsp1. TGF- β signalling is increased in scribble-knockdown cells cultured alone and with normal MDCK cells, but TGF- β signalling is not involved in cell competition. I will now discuss the implications of these findings in a wider context.

6.2 Implications for cell competition

It has been proposed that cell competition may be caused by cells competing for extra-cellular growth factors (Moreno et al., 2002). In mammalian cell culture, cells are grown in a very rich medium containing a very high level of growth factors and mitogens. Due to the abundance of extra-cellular factors in a cell culture model of cell competition, it seems unlikely that cells are eliminated due to a limiting supply of extra-cellular survival factors. It has been shown in *D. melanogaster* that clones of *scribble* mutant cells are not rescued by increased growth factor signalling and have increased endocytosis (Brumby and Richardson, 2003). Together, these data suggest it is unlikely that elimination of scribble depleted cells from a normal epithelium is due to a failure to capture sufficient extra-cellular, life supporting signalling molecules.

Depletion of scribble protein causes a loss of epithelial morphology in *D. melanogaster* and mammalian epithelial cells (Bilder and Perrimon, 2000, Qin et al., 2005b). In *D. melanogaster* deletion of Lgl, another member of the Scribble

complex, results in a loss of epithelial morphology and gives a cell competition phenotype (Bilder et al., 2000b, Tamori et al., 2010, Menendez et al., 2010). Despite the importance of scribble in epithelial morphology in *D. melanogaster*, rescue of polarity defects in scribble mutant clones does not suppress the induction of cell death in these cells (Leong et al., 2009). Also, scribble-knockdown MDCK cells surrounded by normal MDCK cells have no identified defects in epithelial polarity; in fact, the neighbouring normal MDCK cells seem to rescue some cell-autonomous defects of E-cadherin localisation in scribble-knockdown MDCK cells. Therefore, it seems that loss of epithelial polarity in cells lacking scribble is not the cue to neighbouring normal epithelial cells that they should be removed from the epithelium.

A difference in metabolic rate has also been proposed by some to be the cause of cell competition (Johnston, 2009). Scribble has not previously been shown to have any metabolic role, and the role of metabolism has not been investigated in scribble related cell competition. This would be an interesting question to address in the future. In the first discovery of cell competition, and in some reports since, cell competition is described as the removal of slow growing cells (Menendez et al., 2010, Morata and Ripoll, 1975, Johnston, 2009). I have shown that there is no difference in the proliferation rate between scribble-knockdown MDCK cells and normal MDCK cells; therefore, different proliferation rate cannot be the reason that scribble-KD cells die.

p38 activity is increased in scribble-KD cells, and inhibition of p38 activity significantly inhibits death of scribble-KD cells. p38 is activated due to many forms of cellular stress; what kind of stress is occurring in scribble-KD cells is not known. Interestingly, the scribble binding protein β -PIX has been shown to enhance p38 activation in the absence of any other stimuli (Lee et al., 2001). What role β -PIX may have in cell competition induced by lack of scribble is not known. The role of β -PIX in epithelial cells is not known and it has never been studied with respect to cell competition. It would be interesting to understand the localisation of β -PIX in scribble-knockdown cells alone and when surrounded by normal cells.

Flower has been identified as a critical protein in cell competition in *D. melanogaster*. A particular isoform of Flower is upregulated in outcompeted cells in all types of cell competition, and this signals to neighbouring cells that the cell should die. This is downstream of growth factor deprivation in some models of cell competition, so it is not the initial recognition factor in cell competition (Rhiner et al., 2010). Flower is conserved in mammals, so it is very likely that it has a similar function to that described in *D. melanogaster*.

6.3 Is cell competition the same in MDCK cells and *D. melanogaster*?

In *D. melanogaster* it has been shown that cell competition is not an entirely epithelial cell phenomenon, and that the immune system is also involved (Cordero et al., 2010). Data presented in this thesis and by others has shown that cell competition can occur solely due to interactions between epithelial cells (Tamori et al., 2010). Also, experiments in *D. melanogaster* are in a developing organism, with a complex background of signalling gradients. Consequently, cell competition has been described as a mechanism to eliminate weak cells to ensure the best fitness of the organism (Johnston, 2009). Experiments in mammalian cell culture show that cell competition can occur outside of a developmental context, and that cell competition could simply be a cell biology problem, not a developmental biology problem.

6.4 Implications of the death of scribble-KD cells on tumourigenesis

Data presented in this thesis identifies scribble as the first tumour suppressor protein shown to be involved in cell competition in mammalian cells. It is theorised that the loss of scribble protein in one cell in a normal epithelium could be a model of the first stage of tumourigenesis. In this case, the induction of cell death in this cell would prevent any further tumourigenesis. Therefore, in order for loss of scribble to contribute to the progression of a tumour, the tumour must first have acquired one of the characteristics essential for all

tumours, namely the evasion of apoptosis. (Hanahan and Weinberg, 2000). This means that loss of scribble cannot be the first transformative step in cancer. Experiments in *D. melanogaster* and mammalian cells have shown that when scribble is lost in cells that are already transformed by expression of an oncogene, then the tumourigenic ability of the cell is greatly increased (Pagliarini, 2003, Brumby and Richardson, 2003, Dow et al., 2008, Zhan et al., 2008). One of these studies shows that loss of scribble in cells that over-express Myc inhibits apoptosis caused by over-expression of Myc and allows formation of large tumours (Zhan et al., 2008). These data are from experiments that only look at cell-autonomous signalling, i.e. the transformed cells are never in contact with non-transformed epithelial cells. It would be very interesting to study the interaction of Myc over-expression with the loss of scribble in a more physiological setting, where the interaction of non-transformed cells with those that have lost scribble expression and over-express Myc is maintained. From a cancer perspective, it is also important to understand if cell competition interactions only occur when cells that have no scribble expression contact epithelial cells, or whether some interaction can also occur between epithelial cells lacking scribble and non-transformed stromal fibroblasts.

6.5 Future perspectives

There are many unanswered questions regarding data presented in this thesis. I believe that it is important to repeat data presented in this thesis in another epithelial cell line, in particular MCF10A cells. There are several reasons for this. First, repeating this data in another cell line would add certainty to conclusions drawn from this work. Secondly, there have been reports that knockdown of scribble may have different effects in MDCK cells and MCF10A cells; in particular, knockdown of scribble in MCF10A has been reported to have no effects on cell morphology (Qin et al., 2005b, Zhan et al., 2008). This would help understand if the morphological changes associated with scribble-knockdown in MDCK cells is related to their death when in contact with normal MDCK cells. Finally, working with a human cell line such as MCF10A makes screening with small molecule and

shRNA libraries and microarrays much more feasible. These approaches are likely to be required to identify the mechanism by which scribble-knockdown cells and normal MDCK cells initially recognise their difference. Work in *D. melanogaster* has shown that such a screening approach can be very effective.

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